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EXPERIMENTAL CELL RESEARCH

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VOLUME 13 · OCTOBER 1957 · NUMBER 2

AP

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N. Y.

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NUCLEIC ACID CONTENT AND ULTRAVIOLET SUSCEPTIBILITY OF *TETRAHYMENA PYRIFORMIS*[1]

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Received December 15, 1956

The discovery of a method for synchronizing division in *Tetrahymena* by heat treatment [13, 14] has provided interesting material for a study of the relationship between ultraviolet (UV) susceptibility and content of nucleic acid, since nucleic acids and their precursors accumulate in such heat-treated individuals. The nucleic acids and other cellular constituents of tetrahymenas are partitioned gradually among the offspring during the synchronized divisions subsequent to heat treatment, again providing tetrahymenas with contents of nucleic acid and its precursors different from that of the tetrahymenas sampled before heat treatment. This paper records studies on the UV radiation susceptibility of the tetrahymenas before heat treatment and after the first division subsequent to heat treatment, correlated in each case by their content of deoxyribonucleic acid (DNA), deoxyribonucleosides and deoxyribonucleotides, and ribonucleic acid (RNA). For this purpose UV radiations of wavelengths 226 and 265 mμ were used, the first because such short radiations are absorbed so superficially and might be confined to the cytoplasm, the other because it is absorbed so strongly by the nucleic acids. The results indicate that the tetrahymenas which have accumulated nucleic acids or their precursors during heat treatment are more resistant to UV radiations than those not subjected to heat treatment.

MATERIAL AND METHODS

Cultures of *Tetrahymena pyriformis* (*geleii*) strain W [5, 7], obtained through the courtesy of Drs. Kidder and Dewey, were grown axenically on the tryptone-acetate medium described in another paper [4]. Division in a culture was synchronized by the heat treatment technique of Scherbaum and Zeuthen [13], exposing them for eight hours to alternate temperatures of 34 $\pm$0.5°C and 29 $\pm$0.5°C for 30 minutes

[1] Supported in part by funds made available by contract C-1799(C3R) M and G, with the U.S. Public Health Service. The authors are indebted to Dr. Erik Zeuthen for a critical reading of the manuscript and helpful suggestions.

each. Cultures were grown as follows. From a 10 ml stock culture transferred frequently enough so as to always be in logarithmic phase, one individual was isolated into 10 ml of fresh nutrient medium and incubated at 20°C for four days prior to use in an experiment. On the fourth day, the vigorously growing culture was poured into a 1500 ml Ehrlenmeyer flask containing 100 ml of fresh nutrient medium. To this culture, which was incubated for a day at 20°C, 50 ml of fresh nutrient medium was again added two hours prior to the beginning of an experiment, to insure adequate nutrient. At the beginning of an experiment, one-fourth of the culture was set aside to serve as the source of tetrahymenas for the controls and for irradiation without heat treatment. The remainder of the culture was centrifuged, the supernatant fluid was removed by suction, and the tetrahymenas were suspended in 70–90 ml of fresh nutrient medium to make a suspension containing 1 to 3 $\times 10^4$ individuals per ml. The culture was then poured into a sterile, dry, one liter Ehrlenmayer flask and placed in the heat treatment bath.

A culture was prepared for irradiation by centrifugation and washing twice in 50 ml of sterile water. About 100 tetrahymenas suspended in water were added to the quartz irradiation cell. For purposes of plotting, the end of each irradiation period was always taken as zero time.

The radiations of a quartz mercury arc were resolved by a quartz monochromator to give UV radiations of wavelengths 226 and 265 mμ, the intensity of which was measured with a thermopile calibrated against standard lamps. The tetrahymenas were irradiated and handled in the same manner as described elsewhere [4]. In brief, after irradiation they were isolated in hanging drops of about 2 lambda volume and kept at 20°C in a room lighted with red lamps. The number of individuals in a drop was determined periodically and from this the number of hours elapsing until a tetrahymena divided once (T_1), twice (T_2) or three times (T_3) was determined. Similar counts were made for untreated controls isolated at the same time as the experimental cultures for the first (T_{1C}), second (T_{2C}) or third (T_{3C}) divisions after subculture.

For photoreversal of UV injury the tetrahymenas in hanging drops were placed 31 centimeters from a bank of three fluorescent lamps, two 40 watt Sylvania cool-white and one 40 watt Westinghouse warm-white lamp within 20 minutes after the end of exposure to UV radiations. Counts were made on these as in the other cases.

To determine the delay in division induced by UV radiation, the time for the control to divide once, twice or three times was subtracted from the time required for the UV-treated animals to divide the same number of times (i.e., $T_1 - T_{1C}$, $T_2 - T_{2C}$, $T_3 - T_{3C}$). When UV-treated tetrahymenas had divided only two times by the end of 21 days, the time required to reach the third division was arbitrarily assigned a time which exceeded the last counting time. The division delay for irradiated tetrahymenas subjected to visible light to photoreverse the UV injury, was determined in a similar manner.

Tetrahymenas for nucleic acid determinations were from aliquots of cultures maintained continuously in logarithmic phase. They were resuspended in fresh medium at a density of 1 to 3 $\times 10^4$ individuals per ml prior to heat treatment. Sample 12 ml aliquots of the culture were withdrawn periodically from a culture flask and placed in separate test tubes (15.5 $\times$ 1.5 cm) in which all further extractions were performed. From the centrifuged tetrahymenas, after precipitating with 0.3 *M* trichloroacetic acid (TCA) in the presence of albumin, phospholipids were removed

from each sample by adding 5 ml of 95 per cent ethanol and ethyl ether (3:1) to the sample, heating for 5 minutes to the boiling point of the mixture and centrifuging and pouring off the supernatant fluid. The precipitate remaining after pouring off the ethanol-ether, was washed once with 5 ml of 0.3 *M* TCA. Nucleic acids were then extracted from the precipitate by the Hershey, Dixon and Chase [8] adaptation of the Schmidt and Thannhauser [15] and Schneider [16] procedures. First RNA was extracted from the precipitate by hydrolysis in 1 ml of 1 *N* NaOH for 18 hours at 20–24°C, at the end of which time, 1 ml of 1 *N* HCl and 0.2 ml of 3.0 *M* TCA were added to precipitate the unhydrolyzed DNA and protein. To the supernatant fluid, poured into a separate test tube, was now added a 1 ml of 0.3 *M* TCA washing of the DNA and protein precipitate. To a 1 ml aliquot of the RNA extract was added 0.15 ml of 10 per cent orcinol (MacKay Brand, recrystallized twice from benzene) and 2.5 ml of 0.033 per cent $FeCl_3$ dissolved in concentrated HCl [12]. The tubes, capped with glass marbles and shaken, were placed in boiling water for 20 minutes. After cooling under running tap water the optical density of the solution at 565 mμ was determined with a Beckman Spectrophotometer. Experiments were performed in such a manner that duplicate experimental and control determinations could be completed within 30 minutes from the time they were removed from the boiling water bath. The RNA standards (Delta Chemical Works, New York) and the blank were made up in 0.3 *M* TCA.

The DNA was extracted from the DNA-protein precipitate following the removal of RNA which was described above, by two successive extractions at 90°C for 15 minutes with 1.5 ml of 0.3 *M* TCA in each case, the supernatant fluid being collected in another tube for testing. The indole method of Dische [6], as modified by Ceriotti [3], was used to determine DNA, with the exception that the samples used were just half as large, since 0.5 ml microcuvettes (Pyrocell Mfg. Co., N.Y.) were used. The DNA standards (Schwartz Lab. Inc., New York, N.Y.) and the blank were made up in 0.3 *M* TCA. The optical density of the solutions at 565 mμ was determined with a Beckman Spectrophotometer.

Bactoassay of the DNA was performed by the method of Hoff-Jørgensen [9] with slight modifications. *Thermobacterium acidophilus* R-26 Orla Jensen (ATCC 11506), which requires the deoxyribonucleic linkage present in deoxyribonucleosides and deoxyribonucleotides for its growth and therefore grows in proportion to the content of such compounds in the medium, was maintained by weekly transfers in peptone-supplemented basal medium. For assay of DNA and its precursors, a bacterial culture grown for 24 hours in the supplemented basal medium was centrifuged and resuspended in sterile double distilled water for 10 hours prior to use. A 15 ml aliquot of the *Tetrahymena* culture to be tested was withdrawn at each sampling time, centrifuged, and all but 2 ml of the nutrient medium was then withdrawn by suction, after which the tetrahymenas were washed two more times in 50 ml of water each time. After the final washing, water was added to make a 7 ml cell suspension of which 2.5 ml was added to each of 2 test tubes. Counts of the number of individuals per ml were then made so that the deoxyribonucleosides and deoxyribonucleotides could be calculated on a per cell basis. To each of the 2 test tubes enough 5 *N* NaOH was added to give the final NaOH concentration recommended by Hoff-Jørgensen. After the tubes had been boiled and cooled to room temperature, a solution containing 0.31 *M* maleic acid and 0.022 *M* magnesium sulfate was added to give the final re-

commended concentrations. To one of each pair of tubes, stored at 5°C until all samples from one experiment had been collected, 0.1 ml of 1 mg per ml of deoxyribonuclease (Worthington Biochem. Corp., Freehold, N.J.) was added to degrade the cellular DNA, while to the other tube 0.1 ml of water was added. All were incubated for 20 hours at 34°C.

The assay tubes (8 × 75 mm) contained 0.5 ml of basal medium (pH 6.7) plus the desired amount of unknown or standard thymidine (Nutritional Biochem. Corp., Cleveland, Ohio) solution and distilled water to make 1 ml. The tubes, capped with aluminum foil and shaken to ensure mixing of their contents, were autoclaved at 15 lbs and 120°C for 10 minutes. After the tubes had cooled, 0.1 ml of the previously prepared bacterial suspension was added to each tube except the blanks and incubated for 36 hours at 34°C. For each sample duplicate determinations were made of the amount of bacterial growth supported by the compounds with deoxyribonucleic linkage, with a new standard for each experiment. The population density of the bacteria was determined by the transmission (and optical density) at 650 mμ on a Beckman DU Spectrophotometer, using the 0.5 ml micro-cuvettes.

EXPERIMENTAL RESULTS

Accumulation of DNA and RNA during heat treatment

In Table I are summarized the amounts of RNA and DNA present per tetrahymena at five different times preceding, during, and after heat treatment. A small increase in both RNA and DNA is observed soon after heat treatment has begun and by the end of heat treatment the RNA (row 1) has increased to 1.33 that in the untreated individuals while the DNA has increased to between 1.82 (row 2) to 2.35 times that present in the untreated controls.[1] The value for DNA (row 3) depends upon the method used for the determination, the bactoassay method giving a higher value than the indole method, because it tests not only the degradation products from DNA but also the nucleic acid precursors containing the deoxyribonucleic linkage. The first synchronous division after heat treatment reduces the DNA to the amount found in controls, while the RNA is not reduced to this level until after the second synchronous division. The datum in column C, row 4 is

[1] Zeuthen, Scherbaum and Hoff-Jørgensen [17, 9] found a four-fold increase of DNA per *Tetrahymena* at the end of heat treatment. They employed the bacto-assay method which tests for the presence of free deoxyribonucleotides, deoxyribonucleosides, plus those appearing from degradation of DNA. When the same technique was attempted here, individuals at the end of heat treatment had only 2.35 times more of all of these than before treatment. The reason for the discrepancy is not known, but it may be a question of the precise conditions under which the tetrahymenas were grown and treated with heat. If Zeuthen *et al.* provided conditions in which the tetrahymenas grew to 3 to 4 times normal size while those in the present experiments grew to only 2 to 3 times their normal size, there would be no real discrepancy in results. The possibility of a difference in strain of *Tetrahymena* is also suggested.

TABLE I

Nucleic acids content of Tetrahymena *before, during and after heat treatment.*[a]

Method of determining nucleic acids	A Before heat treatment	B After 3 hours of heat treatment	C End of heat treatment	D After first synchronous division following end of heat treatment	E After second synchronous division following end of heat treatment
1 RNA (orcinol)					
Absolute	1.28×10^{-9}	1.49×10^{-9}	1.71×10^{-9}	1.43×10^{-9}	1.2×10^{-9}
Relative	1.0	1.17	1.33	1.12	0.94
2 DNA (indole)					
Absolute	3.03×10^{-11}	3.28×10^{-11}	5.53×10^{-11}	2.83×10^{-11}	3.05×10^{-11}
Relative	1.0	1.08	1.82	0.93	1.01
3 DNA (bacto-assay, with DNA-ase)					
Absolute	5.05×10^{-12}	—	11.9×10^{-12}	5.84×10^{-12}	6.23×10^{-12}
Relative	1.0	—	2.35	1.15	1.23
4 DNA (bacto-assay, no DNA-ase)					
Absolute	1.86×10^{-12}	—	3.36×10^{-12}	3.19×10^{-12}	1.67×10^{-12}
Relative	1.0	—	1.81	1.71	0.9
5 DNA (row 3 minus row 4)					
Absolute	3.19×10^{-12}	—	8.54×10^{-12}	2.65×10^{-12}	4.56×10^{-12}
Relative	1.0	—	2.67	0.83	1.43

[a] Upper values in the table represent the content of nucleic acid in grams per *Tetrahymena*; lower values represent the ratio of columns B, C, D or E to A of that row. Comparisons can only be made across rows 3, 4 and 5 because of uncertainty of the degree of DNAase action.

interesting because it shows that even when no deoxyribonuclease is added before assaying with bacteria, a high value is obtained indicating that the tetrahymenas have accumulated considerable amounts of deoxyribonucleosides and deoxyribonucleotides which are not partitioned until after the second synchronous division.

To see if any correlation exists between the content of nucleic acids (and their precursors) with sensitivity to UV radiations, the physiological stages of *Tetrahymena* chosen were: before heat treatment, at the end of heat treatment, and after the first synchronous division, because these were deemed most likely to give pertinent information.

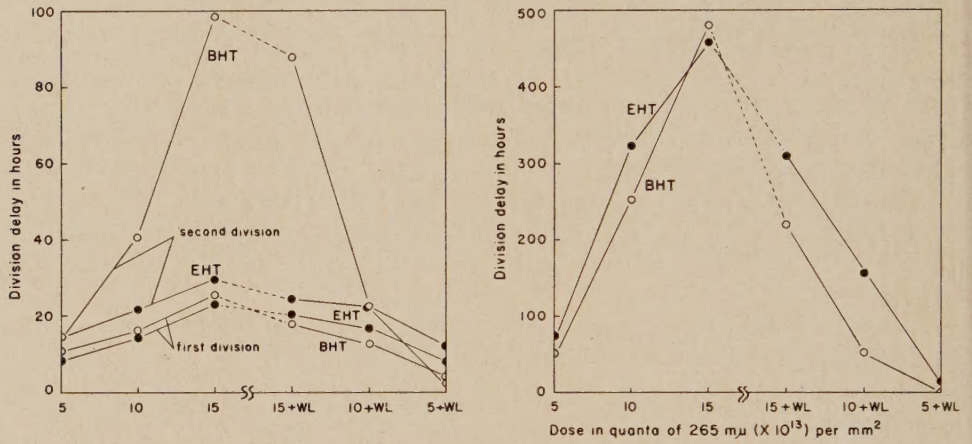

Fig. 1.—*(Left.)* Delay in the first, second, and third post-irradiation divisions of *Tetrahymena* given various dosages of UV at λ 265 mμ. *(Right.)* Delay in the first, second and third post-irradiation divisions after the same dosages but following illumination with white light (WL) for photoreversal. 1A. Delay in the first and second post-irradiation divisions. 1B. Delay in the third post-irradiation division. BHT means before heat treatment; EHT means at the end of heat treatment.

Retardation of division by short ultraviolet radiations (265 mμ)

In all cases division of *Tetrahymena* exposed to UV radiations is delayed as compared to controls; that is, the first, second and third divisions are slower in the irradiated samples than in the controls, indicating some injury. The details of the pattern of injury in *Tetrahymena* have been described elsewhere [4].

For experiments with wavelength 265 mμ the effect of a dosage series of 5, 10 and 15×10^{13} quanta per mm^2 (374, 748 and 1122 ergs per mm^2) was tested on tetrahymenas irradiated in water. In Fig. 1 is shown the difference in resistance between the tetrahymenas before and at the end of heat treatment. For the smaller dosages little difference is seen between the individuals taken before and at the end of heat treatment, but after the delivery of larger dosages of radiations a marked difference between the two is observed, the individuals at the end of heat treatment being much more resistant than those irradiated before heat treatment when delay of the second

division is used as a criterion. This appears to be correlated with the increased content of nucleic acids and precursors in heat-treated tetrahymenas. Since the second division after irradiation shows the differences better than the first or third division, it was chosen as the basis for comparison of the other results presented at this wavelength.

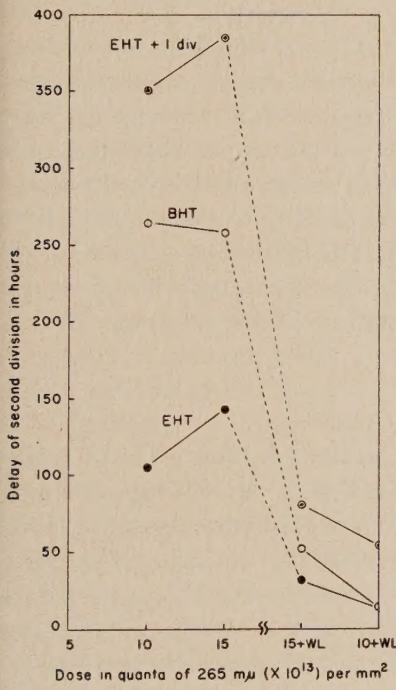

Fig. 2.

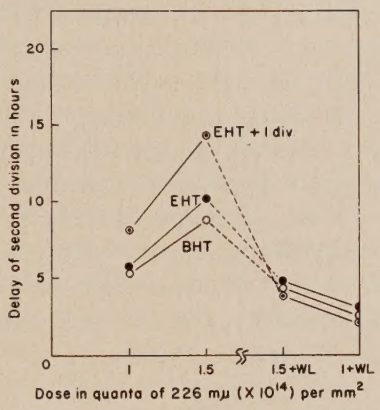

Fig. 3.

Fig. 2.—Delay of the second post-irradiation division of *Tetrahymena* exposed to various dosages of UV of λ 265 mμ. *(Right.)* Delay of the second post-irradiation division after the same dosages of UV but following exposure to white light for photoreversal. BHT means before heat treatment, EHT means at the end of heat treatment and EHT + 1 div. means after the first synchronous division following heat treatment.

Fig. 3.—Delay of the second post-irradiation division of *Tetrahymena* exposed to various dosages of UV of λ 226 mμ. *(Right.)* Effect of the same dosages of UV on the second post-irradiation division but after illumination with white light for photoreversal of UV injury. BHT means before heat treatment, EHT means at the end of heat treatment and EHT + 1 div. means after the first synchronous division following heat treatment.

In the next series of experiments a comparison was made between UV resistance of tetrahymenas before heat treatment, at the end of heat treatment, and after the first post-heat treatment synchronous division. Since about 90 per cent of the tetrahymenas undergo division at 1 to 1.5 hours after being placed at 29°C following the end of heat treatment, an unselected sample was irradiated when the characteristic "eight-shape" of dividing tetrahymenas was observed in the majority of the individuals. The results given in Fig. 2 show that the tetrahymenas which have just divided after heat treatment are more sensitive to UV radiations than individuals not subjected to

heat treatment. These in turn are more sensitive than individuals irradiated at the end of heat treatment but before they had divided.

Retardation of division of Tetrahymena *by very short ultraviolet radiations (226 mμ)*

Because very short ultraviolet radiations of wavelength 226 mμ have such qualitatively different effects on protozoans [2] their effects on divison delay in tetrahymenas before heat treatment, at the end of heat treatment and after the first synchronous division were tested. A unit dose of 1×10^{14} quanta per mm^2 (879 ergs per mm^2) and a dose one and a half times this were used in experiments with this wavelength. In this case also the tetrahymenas were suspended in water since nutrient medium absorbs all of the radiations at this wavelength.[1] As seen in Fig. 3, little difference exists between resistance to UV of wavelength 226 mμ of tetrahymenas before and at the end of heat treatment, but those irradiated after the first synchronous division are somewhat more sensitive.

Photoreversal of UV injury

In Figs. 1, 2, and 3 are given data for the photoreversing effect of white light upon the injury caused by ultraviolet radiation. It will be observed that in almost all cases a considerable degree of photoreversal occurs.[2] It is most striking after irradiation with wavelength 265 mμ, in part because in this case the injury was most marked. The results have several points of interest. First, photoreversal is not a function of the physiological stages tested here since tetrahymenas subjected to UV radiations of wavelengths 265 and 226 mμ before heat treatment, at the end of heat treatment or after the first synchronous division were about equally photoreversed by white light. Photoreversal of UV injury in *Tetrahymena* has been reported in some detail elsewhere [4].

DISCUSSION

It would be interesting to ascertain the cause of the observed differences in UV sensitivity of *Tetrahymena* in different physiological states before and after heat shock. Tetrahymenas were most resistant to UV at the end of heat

[1] Previous trials of the effects of UV of wavelength 265 mμ showed that the radiations retard division of tetrahymenas exposed in either water or nutrient medium to a similar extent. However, because the radiations are absorbed to some extent by the nutrient medium, a larger incident dosage must be applied when nutrient medium is used.

[2] Little photoreversal is shown in Fig. 1A for the higher dosages of UV.

treatment at which time chemical determinations indicate that DNA, DNA precursors and to a lesser extent RNA have accumulated in the individuals. The greater resistance at this stage might well result from the availability of the nucleic acids for partition during division to the daughters of the irradiated individuals. Therefore, even if nucleic acid synthesis were blocked by UV treatment, as has been suggested by Kelner [11], division is not retarded because stored nucleic acids are used.

The present experiments also indicate that UV sensitivity is correlated with DNA stores and not with RNA or nucleotides and nucleosides. After the first synchronous division some excess RNA remains and the precursors of the nucleic acids are still present in almost double their usual amount, yet the sensitivity of *Tetrahymena* to UV has risen many-fold with the partition of DNA between the daughters of the heat-treated individuals.

The tetrahymenas are probably so much more sensitive following the first synchronous division than those tested before heat treatment because they are in a different state. Those tested before heat treatment are in the log phase but probably in many states of preparedness for division—some about to divide, some nearly so and others just after division, etc. The group therefore represents a random sample of a population. After the first synchronous division, the DNA has been partitioned between the two daughters and all the cells are therefore in need of synthesizing more DNA for the next division, probably a critical state. Consequently, they are particularly sensitive to UV, since these radiations inhibit the synthesis of DNA from the immediate precursors [10] so that even in the presence of an excess of deoxyribonucleotides and deoxyribonucleosides in such cells, they remain unusually sensitive to UV.

It is interesting to note that very short UV radiations (226 mμ) affect tetrahymenas before and at the end of heat treatment to essentially the same degree. This suggests that perhaps radiations of this wavelength produce damage elsewhere than by inhibiting nucleic acid synthesis. It was observed that tetrahymenas exposed to this radiation became immobilized quickly, an effect not noted following comparable dosages with longer wavelengths of UV radiations. Since the very short UV radiations are superficially absorbed they may produce their effects in the cytoplasm as indicated by the rapid immobilization of the tetrahymenas. However, since the retardation of division following exposure to radiations of wavelength 226 mμ is subject to photoreversal, and only nuclear effects of UV have been previously found subject to photoreversal [1, 2], it would appear that some nuclear function other than synthesis of DNA is being affected by these radiations.

SUMMARY

1. The extent to which *Tetrahymena pyriformis W* accumulates nucleic acids and their precursors during heat treatment and the influence of such accumulated materials upon ultraviolet (UV) sensitivity of the protozoans was studied.

2. At the end of heat treatment tetrahymenas contain double the amount of the deoxyribonucleic acid (DNA) precursors, deoxyribonucleosides and deoxyribonucleotides, present before heat treatment. The amount is maintained following the first synchronous division, but is reduced to the original amount by the second synchronous division.

3. At the end of heat treatment a tetrahymena has almost double the amount of DNA present before heat treatment. This is partitioned between the daughters at the end of the first synchronous division.

4. At the end of heat treatment a tetrahymena has more ribonucleic acid (RNA) than an untreated individual. The content of RNA is gradually reduced in the synchronous divisions following the end of heat treatment until the normal level is achieved.

5. Division of tetrahymenas is delayed by irradiation, the delay increasing with dosage. Delay of the first division after irradiation is not marked but delay of the second and third divisions following irradiation is more noticeable. The effects of a given dosage of UV of wavelength 265 mμ retards division of tetrahymena more than a like dosage of wavelength 226 mμ. On the other hand, the latter radiations immobilize tetrahymenas much more readily than the former.

6. The second and third divisions subsequent to irradiation with UV are progressively more delayed when tetrahymenas are irradiated with like dosages in the following physiological stages: at the end of heat treatment, before heat treatment, and after the first synchronous division.

7. The greater resistance to UV radiations of tetrahymenas at the end of heat treatment than in other physiological states is correlated with their greater content of DNA in that stage. Thus the UV sensitivity is inversely proportional to the amount of DNA present per cell, and shows a direct relationship with the amount of DNA which a cell must synthesize before division.

8. In spite of almost a doubled amount of deoxyribonucleotides and deoxyribonucleosides in individuals which have undergone the first synchronous division after heat treatment, resistance to UV is very low, indicating

no correlation of UV resistance with these compounds. The block in DNA synthesis may be in the incorporation of these precursors into DNA.

9. Since UV of wavelength 226 mμ has no differential effect on division of tetrahymenas before and at the end of heat treatment, yet it retards division, an effect of UV at a locus other than nucleic acid synthesis is suggested.

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ADAPTATION OF YEAST TO COPPER

XIV. QUALITATIVE CHANGE OF RIBONUCLEIC ACID IN YEAST CULTURED IN COPPER MEDIUM

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Received January 7, 1957

When a yeast strain was treated with the extract (E_R) of its copper-resistant substrain, the copper resistance, as estimated by the ability of forming visible colonies on copper medium, was higher than when the strain was treated with the extract (E_P) itself [11]. Aerobic and anaerobic fermentation of cells treated by E_R was less sensitive to copper than those treated by E_P [14]. The effect of E_R raising the copper resistance of the parent strain was proved to be due to a specific ribonucleic acid (RNA_R) contained in E_R [11–14]. The effect was therefore called the RNA_R-effect [14].

When the parent strain is inoculated in the broth containing $CuSO_4$ at concentrations around 1 m*M*, a rapid growth begins after a lag period of 1 day or so [2, 18, 20]. Cells grown in this way are copper-resistant and give E_R when extracted. Hence the RNA_R formation must begin at some time during the culture of parent cells in the copper medium.

The present paper reports an experiment made to find at what stage of the growth process in the copper medium the RNA_R synthesis begins.

EXPERIMENTAL

The strain of *Saccharomyces ellipsoideus*, as well as the medium and conditions of culture, were the same as in the previous papers [11–14]. The number of cells was counted using a hematimeter. The cells stained by Fink's methylene blue solution were regarded as "dead", and those unstained as "living" [6].

Growth in copper medium.—Parent cells were precultured in liquid MH medium for 2 days. 100 ml of this culture was introduced into 1400 ml of MH medium, and a sterilized solution of $CuSO_4$ was added to it to make the copper concentration 1 m*M*. 300 ml aliquots of this preparation were dispensed in 500 ml Erlenmeyer flasks, and incubated at 30°C. Standing culture was adopted, because, with this, the conspicuous growth of culture, which was due to multiplication of resistant cells, began after a less variable period of inhibited growth than with shaking culture.

Cell numbers per unit volume of culture, in three separate runs of experiment, are given in Table I. Under the conditions used, it took about 5 hours for cell prolife-

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TABLE I

Growth of parent cells inoculated in the MH medium containing 1 mM of $CuSO_4$, and the E_R-effect as represented by the survival ratio, in 1 mM (I) and 1.2 mM (II and III) Cu–MH agar, of parent cells "aerobically" treated by the extract of cells harvested at various periods of incubation.

Time (hr.)	I			II			III		
	Cell conc. (10^6 per ml)		E_R-effect	Cell conc. (10^6 per ml)		E_R-effect	Cell conc. (10^6 per ml)		E_R-effect
	Living	Total		Living	Total		Living	Total	
0	6.28	6.76	87.7	—	—	75.5	—	—	76.5
5	11.0	11.3	—						
10	8.23	11.0	86.4						
18.5				9.48	14.4	73.5			
21							6.00	15.9	79.0
22				7.24	14.9	77.6			
23							4.82	14.0	82.5
24	6.18	9.28	105	5.76	11.7	90.6			
25							6.00	15.2	94.5
27				6.42	11.7	—	6.56	—	97.0
29				7.80	13.2	93.2			
34	6.92	11.2	95.6						
51	49.4	65.8	98.0						

ration to be inhibited by copper. In this period, a slight increase in the cell number was observed, new buds still adhering to their mother cells. Following this period no remarkable change was observed in the total cell number, the number of "living" cells decreasing a little (the descending phase).

Many cells began to sink to the bottom of the flask about 10 hours after inoculation. The staining by methylene blue became less and less distinct as time elapsed. However, in the presence of the dye, it was not difficult to distinguish between the cells which refracted light strongly and those which did not. The number of the former was proportional to the number of viable cells determined by plating, and seems, therefore, to represent, or to be proportional to, the number of "living" cells. The average sizes of "living" and of "dead" cells decreased gradually in the descending phase.

About 24 hours after inoculation, larger cells which refracted light strongly were found attached to clusters which consisted of several "dead" cells of smaller size. Such vigorous cells increased in number with time. They proved to be copper resistant when inoculated into a test copper medium [20]. An analogous growth process was observed also in a synthetic medium containing copper, though the birth of resistant cells was slower in this poor medium than in richer media [17].

Activity of the extract.—Samples were taken from the copper culture at intervals. Cells were washed twice with water, vacuum dried, and 100 mg of dried cells were

extracted with 10 ml of water on boiling water bath. Copper contained in the extract was removed by cation exchange resin (Amberlite IR-120). By adding water the final volume of the preparation was made 10 ml.

Parent cells, gathered from 2-day-old culture on MH agar slant, were suspended in the extract, the gas phase being air. Cells "aerobically" treated in this way were suspended in MH agar before gelation. A portion of this was mixed with a sterilized $CuSO_4$ solution. 20 ml aliquots of the control and of the copper agar were poured into Petri dishes. The colonies were counted after two days of incubation. After this period no visible colonies grew. The percentage of the number of visible colonies growing in the copper plate compared to that in the non-copper plate was called the survival ratio.

Table I shows the survival ratios of the parent strain which were treated by extracts of cells harvested at various periods of copper culture. The survival ratio is low for a certain period, but thereafter increases to nearly 100 per cent. The parent strain which had been furnishing cell extracts of low activity seemed to change to produce active principle during the stay in the copper medium. The survival ratio at zero time of each culture should be taken as the control value, since, in order to obtain this value, the test cells were treated, for the same duration as the latter treatments, with the extract of the parent strain which was to be copper-cultured. It should be mentioned that the following three kinds of cells showed practically the same survival ratios with each other: (*a*) parent cells which had not been treated by any cell extract, namely, those plated directly from the normal culture; (*b*) those treated by $M/15$ KH_2PO_4 during the same time period as the extract treatment; and (*c*) those treated by the extract from the parent strain cells.

It can be seen in Table I that the E_R-effect was only detectable when the number of "living" cells reached its minimum, namely, when large and bright cells were found and the number of cells began to increase. After this period, the E_R-effect continued to be apparent. Hence, it was not later than at this period that the RNA_R-synthesizing system began to work.

DISCUSSION

Due to improvements in the technique of the nucleic acid analysis, different types of yeast RNA have been shown by some authors, although the possible occurrence of artefacts cannot be ruled out. From the ribonucleoproteins separated by isoelectric precipitation, Khouvine *et al.* [7, 8] obtained RNA's differing from each other with regard to their compsition. Miura *et al.* [16] also showed inhomogeneities of RNA by adsorbing it with ion exchange resin and eluting with various ionic strengths of NaCl solution.

Abrams [1] reported that the DNA composition changed along with the growth of *Sacch. cerevisiae*, though no similar result was obtained by Reddi [19] with *Pseudomonas*. Experiments by Dutta *et al.* [4], using *Sarcina lutea*, did not show alterations of DNA and RNA accompanied by growth. According to Dimroth *et al.* [3] the ratio of guanine to adenine of RNA decreased to half its normal value, when the mean generation time of *Torula* increased in the presence of NaN_3. However, they did not refer to the biological meaning of the qualitative change of the RNA.

The present author [11] has found that an extract with E_R-or RNA_R-effects can not only be obtained from the copper-resistant substrain cultured in the copper medium, but also from that cultured in the copper-free medium (to be denoted as $R_{lb(0)}$). Hence, the presence of a significant amount of copper in the medium does not seem to be necessary for the formation of the RNA_R.

It has been shown [15] that RNA of the parent strain has a significantly lower ratio of cytidylic acid to adenine than has RNA of $R_{lb(0)}$ ($P \leq 0.01$ in F-test). Dutta *et al.* [4] claimed that a difference in the base composition of RNA was found between the normal and the streptomycin-resistant *Sarcina* strains. On the other hand, Elson *et al.* [5] reported that the base composition of RNA of sea urchin egg did not vary during 48 hours of embryonic development after fertilization. Knite [9] stated that RNA of mutant plant viruses did not differ from that of the parent strain.

Although it has been revealed that RNA of a copper-trained substrain of yeast differs from that of its parent strain in its base compositon, it is possible that, in the RNA preparation used, the RNA responsible for the E_R effect is contaminated by non-effective types of RNA. Hence it cannot be decided whether the base composition of effective RNA is different from that of non-effective RNA. The composition of RNA_R should be studied by using a preparation of the highest specific activity. The specific activity of the pneumococcal transforming principle was reported to be raised through the separation from other DNA [10].

Although the critical time of the change in RNA could not be accurately determined by the present method, it was found that the synthesis of the RNA_R had begun within 24 hours of keeping the parent strain in the nutrient medium containing copper. Experiments II and III in Table I show that the E_R-effect rose to the maximum in a short time. Hence a rather sudden alteration in the RNA composition seems to have occurred. The E_R-effect was not found when the extract or the RNA from the parent strain was supplemented with copper. Hence the RNA_R does not seem to be formed

in vitro by the chemical reaction of the extract with copper, but seems to be synthesized in cells which have undergone some alterations in the metabolism during culture in the copper medium.

According to Ashida *et al.* [2], the cells having stable copper resistance are missing in the parent population, but they arise *de novo* during the course of culture in the copper medium. This is supported also by the observation described above that the larger bright cells which were copper-resistant began to appear, at a certain period of copper culture, among small cells which formed almost dying clusters.

In studying the conditions of the action of RNA_R, the present author [14] presumed that RNA_R affects the parent cells so as to change the nitrogen metabolism which is not fatally inhibited by copper. The RNA_R which is formed and is working in cells of the resistant strain may possibly be regulating the nitrogen metabolism in that way.

When the parent strain is inoculated in the copper medium, the cell metabolism must be distorted in some manner. It is tempting to assume that such a distorted metabolism eventually results in the switching of part of the RNA synthesis to one producing RNA_R among other ribonucleic acids, and that one of the keys for establishing the stable resistant strain consists of this switching of the RNA-forming system.

SUMMARY

While a strain of *Saccharomyces ellipsoideus* was cultured in the copper medium, cells began to form a type of ribonucleic acid, RNA_R, which was characteristic of the copper-trained strain and had the effect of making its parent strain less susceptible to copper. The RNA_R became detectable in the extract of the copper-cultured cells just at the time when resistant cells were formed growing among surviving parent cells.

The author wishes to express his gratitude to Professor Joji Ashida, Kyoto University, for his encouragement and for valuable discussions. The work was partly supported by Scientific Research Expenditure of the Ministry of Education.

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ELECTRON MICROSCOPIC OBSERVATIONS ON THE CELL-FREE NATURE OF GLOMERULAR BASEMENT MEMBRANE AFTER TREATMENT OF ISOLATED RENAL GLOMERULI WITH TRICHLOROACETIC ACID[1]

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Received January 18, 1957

Renal capillary glomerular basement membrane can be rendered seemingly free of cells either by sonic vibration of isolated glomeruli [2] or by their treatment with 60 per cent (w/w) trichloracetic acid (TCA) at 25°C [1]. The fragmented basement membranes obtained by sonic vibration are optically clear and refractile with the light microscope. There are no recognizable attached portions of cells. The glomeruli, when adequately treated with TCA, maintain their form. There may be distortion of the loops or thickening of the capillary basement membranes but the latter again are optically clear with the highest powers of the light microscope. All the cells apparently have been dissolved by the TCA.

Renal glomeruli, even as vascular tissues in general [3], contain a potent "nephrotoxic" antigen(s), the heterologous antiserum to which is capable of selectively producing a glomerulonephritis in the donor species of animal. The weight of evidence has been that this antigen is an integral part of the capillary basement membrane and is not a component of the cells attached to it [2]. Thus, virtually all of the antigenic material is found in the presumably cell-free basement membrane fraction of sonically vibrated isolated glomeruli. Weight for weight this fraction has about double the amount of antigen as that of whole glomeruli. Likewise, the supposedly cell-free TCA treated glomeruli retain the same degree of antigenicity as the sonically fragmented membranes. Furthermore, Mellors, Siegel and Pressman [4], by the use of fluorescently tagged chicken anti-rabbit globulin antibodies, have shown that rabbit antibodies against kidney and lung of the rat localize in the renal glomeruli of the rat, achieving maximum concentration in the glomerular basement membranes. Nevertheless, it was important to exclude

[1] Supported by a Grant from the U.S. Public Health Service H-1623C5.

the existence of fine cytoplasmic films, particularly endothelial, which might remain adherent to the basement membranes and which might have to be reconsidered as the possible site of the antigen(s). Thus, the lamina fenestrata which normally lines the greater part of the glomerular capillary can only be seen with the electron microscope. It was with this in mind that electron microscopic studies were made of isolated glomeruli treated with TCA.

PROCEDURE

The procedure for the isolation of glomeruli from kidneys, deep frozen at −15°C very soon after removal from dogs, but stored for a variable length of time, has been fully described [2].

The isolated glomeruli were packed by centrifugation and suspended in 60 per cent (w/w) trichloracetic acid. A broad bottomed flask was used to obtain maximum exposure of all glomeruli to the acid. By adjusting the heat from a goose-neck lamp over the flask, the temperature could be maintained at about 25°C. The suspension was gently stirred at frequent intervals. Glomeruli adhering to the walls of the flask were re-immersed in the TCA. Glomeruli so treated become very soft, plastic, almost semiliquid, and translucent. Nuclei and cell cytoplasm slowly dissolve. With more prolonged exposure, the capillary loops expand, the membranes swell and, in time, solution of the membranes likewise occurs. In general, the glomeruli were exposed to TCA for 5 or 6 hours at 25°C on the same day they were prepared. They were then refrigerated at 4°C overnight, but were brought and kept at 25°C for another two or more hours the following day. At this stage, there were no visible cellular components either when examined directly with the light microscope or when samples were removed, drained and washed free of acid and stained with Loeffler's alkaline methylene blue and hematoxylin and eosin. Chemical determinations for desoxyribonucleic acid and ribonucleic acid of aliquots were minimal or nil. Care had to be taken in bringing these very soft plastic glomeruli into a saline suspension since they will adhere and form a gummy mass. To avoid this, it was necessary to centrifuge the TCA suspension lightly, withdrawing the supernatant. At first, the glomeruli were washed with 60 per cent TCA by centrifuging and withdrawal of the supernatant. Following this, the glomeruli were resuspended in progressive dilutions of TCA. The glomeruli were allowed to settle and the supernatant was withdrawn by suction. This procedure was carried out until physiologic saline could be used. These glomeruli were more opaque and smaller than when first isolated.

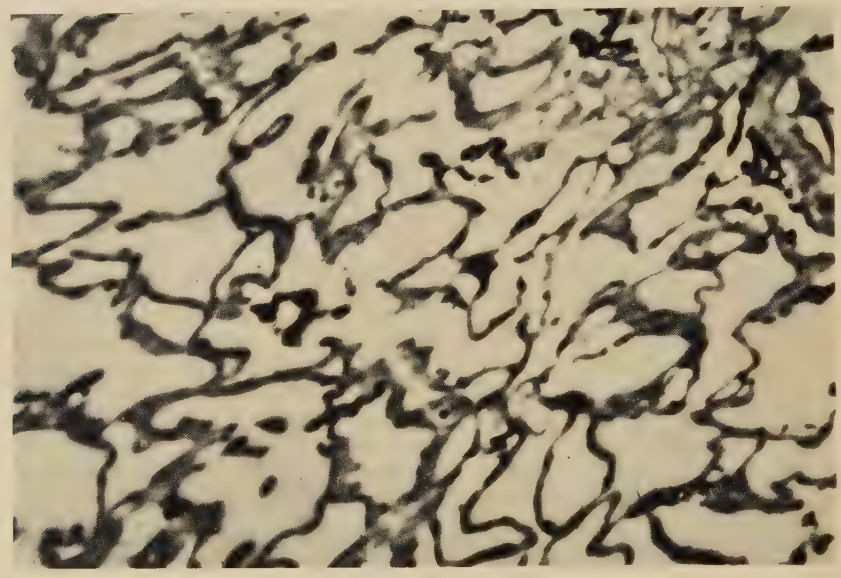

Fig. 1.—Isolated canine renal glomeruli treated with 60 per cent trichloracetic acid (×4000). The capillary basement membranes are of varying thickness with no clearly recognizable cells or cellular membranes.

The treated glomeruli were fixed for 15 minutes in 1 per cent osmic acid buffered with veronal-acetate at pH 7.6 according to the method of Palade [5]. Dehydration was carried out in progressive fashion by resuspension and centrifugation of the glomeruli through 25, 50, 70, 80, 95 and 100 per cent ethyl alcohol at intervals of 30 minutes. After 3 changes in absolute alcohol, the glomeruli were brought into an equal mixture of absolute alcohol and methacrylate. Following 3 changes in *N*-butyl methacrylate, they were imbedded in a mixture of 10 per cent methyl methacrylate and 90 per cent *N*-butyl methacrylate. The mixture was polymerized in an oven at 60°C overnight. Sections were cut at $\frac{1}{40}$–$\frac{1}{20}$ μ on a Porter-Blum microtome and directly examined in an RCA electron microscope (EMU-2a).

RESULTS

The glomeruli treated with TCA present no clearly recognizable cells or cellular membranes. The basement membranes are irregularly widened and seemingly thickened. There are points of disruption of these membranes with often tapering of the ends. The denser structures, seen in the accom-

panying photograph, seem to be portions of basement membrane. They are, in part, in alignment and continuous with the membranes. It is possible that they may be the result of excessive shrinkage associated with the removal of the TCA. It is not very likely that they represent the pedicels of visceral epithelial cells since these structures, as observed electron microscopically, are disrupted and detached in the isolated glomeruli prior to treatment with TCA.

SUMMARY

Sixty per cent trichloracetic acid (w/w) at 25°C is effective in dissolving the cells and cellular membranes of isolated canine renal glomeruli, leaving a somewhat altered basement membrane.

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THE RELATION OF PROTOPLASMIC GELATION TO OXIDATIVE PROCESSES[1]

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Received January 31, 1957

For the general physiologist and also for the biochemist interested in vital phenomena, one of the most interesting and at the same time one of the most difficult of all problems is the question as to the relation between the activity of a living system and the rate of oxidation going on within it. For example every muscle physiologist is interested in knowing how the chemical reactions of a muscle are harnessed to do mechanical work. The distinguished biochemist Meyerhof [9] was intrigued by this problem and in the opening sentence of his classic book, *Die chemischen Vorgänge im Muskel*, he states, "Das Ziel der Muskelphysiologie ist die Beantwortung der Frage, wie die chemischen Prozesse im Organismus mechanische Arbeit leisten; es ist daher ein Problem der allgemeinen Physiologie".

In general, biochemists have thought that when a tissue is roused to activity, this effect is due to some action of stimulating agents on oxidative enzyme systems. But they have never attempted to explain how heat, cold, uneven pressure, ultraviolet radiation, or an electric shock can produce such an effect on an enzyme system. One of the arguments which has influenced biochemists is the belief that anesthetic and narcotic agents, that is to say, agents which prevent tissues from being aroused, act directly by inhibiting one or another oxidative enzyme system. But the evidence for this point of view has largely fallen away, as can clearly be seen from the recent review of Hunter and Lowry [7].

The present paper deals with the relation between the viscosity changes and the oxidative changes which occur during cell division. The mitotic gelation which is essential for the division process is thought to be associated with a change of SH groups to S–S groups. This oxidative process may initiate or trigger other oxidative processes within the cell.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service. The work was done at the Marine Biological Laboratory, Woods Hole, Mass., U.S.A.

MATERIALS AND METHODS

In the following experiments eggs from the worm, *Chaetopterus pergamentaceus*, the sea urchin, *Arbacia punctulata*, and the surf clam, *Spisula solidissima*, were used. Eggs and sperm of *Chaetopterus*, *Arbacia*, and *Spisula* were prepared for experimentation using the same techniques described by Heilbrunn and Wilson [5], Wilson [15] and Allen [1], respectively.

The solutions used in these experiments were prepared daily. 0.01 molar solutions of potassium cyanide and of sodium azide in sea water were prepared and the pH of these solutions was adjusted to 8.0 ± 0.2 with 0.1 or 0.01 normal HCl. Dilutions when desired were made from these solutions. Solutions of *p*-chloromercuribenzoic acid (obtained from the Delta Chemical Works, Inc. and from the Mann Research Laboratories, Inc.) were prepared by dissolving the solid in a small volume of normal NaOH and then adding distilled water to make the concentration 10^{-2} molar. Sea water was then added to this solution until the concentration of *p*-chloromercuribenzoic acid was either 10^{-3}, 10^{-4}, or 10^{-5} molar. The pH of the final solution was brought to 8.0.

Protoplasmic viscosity (relative) determinations were made by the centrifuge method [5, 15]. The term centrifuge value is used as a measure of the magnitude of relative viscosity and is defined as the number of seconds of centrifugation at a given centrifugal force necessary to move the cytoplasmic inclusions into a certain fraction of the egg. In the eggs of *Arbacia* and of *Spisula* the end point used for the centrifuge value was the formation near the centripetal pole of the egg of a clear area in the cytoplasm, the hyaline zone, about $\frac{1}{3}$ the diameter of the egg, and in the egg of *Chaetopterus* the end point used was the formation of a hyaline zone plus a definite accumulation of lighter (presumably fat) granules at the centripetal pole of the egg. In studying *Arbacia* eggs, the width of the hyaline zone for a given time of centrifugation was sometimes used as a measure of viscosity.

All experiments were done at a temperature of 21°C.

RESULTS

In the untreated egg of *Chaetopterus*, there is an increase in protoplasmic viscosity, the mitotic gelation, just prior to the formation of the mitotic spindle. At 21° the mitotic gelation occurs about 28 minutes after fertilization. The viscosity (centrifuge value 14) remains high for about 10 minutes and then decreases to its original value (centrifuge value 7). During the time that the viscosity is reverting from a high value to a low value the mitotic spindle forms [5]. *Chaetopterus* eggs fertilized in sea water and transferred to 10^{-2} *M* KCN two minutes after insemination do not undergo the mitotic gelation but remain in a fluid condition. Moreover, these eggs do not cleave. The results of the effect of KCN on the relative protoplasmic viscosity and on the cleavage of *Chaetopterus* eggs is summarized in Table I. Although 10^{-2} *M* KCN was

TABLE I

The effect of cyanide solutions on protoplasmic viscosity and cleavage of Chaetopterus *eggs.*

The eggs were transferred to the cyanide solution two minutes after insemination.

Concentration of KCN molarity	Viscosity in terms of centrifuge value 30–34 minutes after insemination		Per cent of eggs cleaved	
	Control	Treated	Control	Treated
0.01	14	8 or less	99	0
0.01	14	7	100	0
0.01	14	8 or less	100	0
0.01	14	8 or less	97	0
0.01	14	8 or less	98	0
0.005	14	8 or less	98	0

TABLE II

The effect of 0.005 M KCN on the cleavage of Chaetopterus *eggs when the eggs were immersed in it at various times after insemination.*

The cleavage time of the control eggs was 54 minutes and the percentage of eggs cleaved was 100.

Min. after insemination when eggs were immersed in KCN	Per cent of eggs cleaved	Min. after insemination when eggs were immersed in KCN	Per cent of eggs cleaved
5	0	30	25
10	0	35	23
15	1	40	68
20	0	45	89
25	11	50	94

generally used in these experiments, similar effects could be obtained with 5×10^{-3} *M* KCN, but 100 per cent inhibition of cleavage was not always obtained in this concentration.

The effect of KCN on *Chaetopterus* eggs is reversible, that is to say, when the eggs are removed from the KCN at the time of cleavage of the control eggs and placed in sea water the division process progresses and the eggs cleave. The percentage of eggs which cleave following removal from KCN is variable, depending on how well the KCN is washed from the eggs. Thus in two experiments picked at random the percentage of cleavage was 100 and 33.

TABLE III

A comparison of the relative viscosity of the protoplasm of fertilized Arbacia *eggs in sea water with the viscosity of the protoplasm of fertilized eggs in 0.001 M KCN in sea water.*

The more viscous the egg protoplasm, the narrower the hyaline zone, H.Z., which appears when the egg is centrifuged. All centrifuge tests were made for 15 seconds at a force of approximately 9600 times gravity. Cleavage time for the control eggs was 58 minutes. 92 per cent of the control eggs cleaved. None of the eggs treated with cyanide cleaved.

Min. after insemination	Control eggs in sea water	Eggs in 0.001 *M* KCN
5	of 50 eggs, 50 have H.Z. $^1/_3$ diameter of egg	of 50 eggs, 50 have H.Z. $^1/_3$ diameter of egg
10	of 50 eggs, none has H.Z.	of 50 eggs, 36 have H.Z. $^1/_3$ diameter of egg
15	of 50 eggs, none has H.Z.	of 50 eggs, 19 have H.Z. $^1/_3$ diameter of egg or less
20	of 25 eggs, 8 have H.Z. $^1/_8$ to $^1/_4$ diameter of egg	of 50 eggs, 8 have H.Z. $^1/_8$ to $^1/_4$ diameter of egg
25	of 50 eggs, 46 have H.Z. $^1/_4$ diameter of egg or less	of 50 eggs, 4 have very narrow H.Z.
30	of 50 eggs, 47 have H.Z. $^1/_4$ diameter of egg	of 50 eggs, 3 have very narrow H.Z.
35	of 25 eggs, 25 have H.Z. $^1/_4$ to $^1/_3$ diameter of egg	of 50 eggs, none has H.Z.
40	of 25 eggs, 25 have H.Z. $^1/_4$ diameter of egg	of 50 eggs, none has H.Z.
45	of 50 eggs, 50 have H.Z. $^1/_3$ to $^1/_2$ diameter of egg	of 50 eggs, none has H.Z.
50	of 50 eggs, 50 have H.Z. $^1/_3$ to $^1/_2$ diameter of egg	of 50 eggs, none has H.Z.
55	of 50 eggs, 3 have H.Z. $^1/_3$ diameter of egg	of 50 eggs, none has H.Z.

A few experiments were carried out to determine the percentage of cleavage of eggs placed in KCN at various times after insemination. The results of one experiment are presented in Table II. However, in view of the fact that the rate of penetration of KCN into *Chaetopterus* eggs is not known it is difficult to interpret these results. The results might mean that the most sensitive stage or stages of mitosis with respect to KCN occurs prior to 40 minutes after insemination, or the results might simply reflect the rate of penetration of KCN into the egg. Probably however the rate of penetration of KCN into the egg is rather rapid. This much is clear—the greatest inhibition of cleavage occurs when the eggs are immersed in KCN prior to 40 minutes after insemination.

TABLE IV

The effect of 0.01 M sodium azide on protoplasmic viscosity and cleavage of Chaetopterus *eggs.*

The eggs were immersed in the azide solution two minutes after insemination. The viscosity value for the control eggs at 30 minutes after insemination is 14. The cleavage time of the control eggs is 56 minutes.

Centrifuge value (viscosity) 30 min. after insemination	Percentage of eggs cleaved in azide	Percentage of control eggs cleaved
8 or less	21	99
8 or less	0	100
6	0	100

In *Arbacia* eggs 10^{-3} *M* KCN inhibits cleavage, but the effect of KCN on protoplasmic viscosity is quite different from that on the protoplasmic viscosity of *Chaetopterus* eggs. In the egg of *Arbacia*, KCN enhances the magnitude of the mitotic gelation and also prevents the reversal of gelation which normally occurs in the mitotic process. For the normal *Arbacia* egg the centrifuge value at the peak of the mitotic gelation is greater than 20 and less than 25, but in *Arbacia* eggs treated with KCN the centrifuge value rises to 30. Moreover, in the treated eggs the viscosity does not begin to increase as soon as it does in the control eggs. Actually there is a delay of some 5 or more minutes in the time of appearance of the mitotic gelation.

The results of a typical experiment are presented in Table III. KCN was generally used in a concentration of 10^{-3} *M*, but similar effects could be obtained with a 10^{-5} *M* solution.

As in the *Chaetopterus* egg, the effect of KCN on *Arbacia* eggs is reversible. For example, in one experiment when eggs were transferred from KCN to sea water at the time of first cleavage of the control eggs, 84 per cent of the eggs which had been transferred continued the division process and cleaved and in another experiment 90 per cent of such eggs cleaved.

Sodium azide (10^{-2} *M*) has effects similar to cyanide on the egg of *Chaetopterus*. Azide inhibits cleavage and prevents the mitotic gelation (see Table IV which gives the results of 3 experiments). The effect of azide on the egg of *Chaetopterus* is reversible. For example in two experiments picked at random, 98 per cent of the eggs removed from azide continued the division process and cleaved and in the other experiment 30 per cent of the eggs cleaved.

In the egg of *Arbacia*, sodium azide has effects similar to those of cyanide. In *Arbacia*, azide inhibits cleavage, enhances the mitotic gelation, and in-

TABLE V

A comparison of the relative viscosity of the protoplasm of fertilized Arbacia *eggs in sea water with that of fertilized eggs in 0.01 M sodium azide.*

The more viscous the egg protoplasm, the narrower the hyaline zone, H.Z., which appears when the eggs are centrifuged. Centrifuge tests before 23 minutes after insemination were made for 15 seconds and those after 23 minutes after insemination were made for 20 seconds at a force approximately 9600 times gravity. Cleavage time for control eggs was 55½ minutes. 99 per cent of the control eggs cleaved. None of the eggs treated with sodium azide cleaved.

Min. after insemination	Control eggs in sea water	Eggs in 0.01 *M* sodium azide
7	of 25 eggs, 25 have H.Z. 1/4 to 1/3 diameter of egg	of 25 eggs, 25 have H.Z. 1/3 to 1/2 diameter of egg
11	of 25 eggs, none has H.Z.	
14	of 25 eggs, none has H.Z.	of 25 eggs, 25 have H.Z. 1/4 diameter of egg
18½	of 25 eggs, none has H.Z.	of 25 eggs, none has H.Z.
23	of 25 eggs, none has H.Z.	of 25 eggs, none has H.Z.
28	of 25 eggs, none has H.Z.	of 23 eggs, none has H.Z.
33		of 25 eggs, none has H.Z.
37	of 25 eggs, 25 have H.Z. 1/3 diameter of egg	of 25 eggs, none has H.Z.
42	of 25 eggs, 25 have H.Z. 1/3 to 1/2 diameter of egg	of 25 eggs, none has H.Z.
47	of 25 eggs, 25 have H.Z. 1/2 diameter of egg	of 25 eggs, none has H.Z.

hibits the reversal of the mitotic gelation. In azide also there is a delay in the onset of the mitotic gelation. The results of a typical experiment are presented in Table V. The effect of azide on the *Arbacia* egg is reversible. In one experiment 96 per cent of the eggs cleaved when removed from the azide to sea water at the time of cleavage of the control eggs and in another experiment 98 per cent of the eggs cleaved when transferred at the time of cleavage from the azide to sea water.

Although at the present time sufficient data necessary to plot a curve for the viscosity changes in the egg of *Spisula* during mitosis have not been obtained, the data indicate that the curve resembles that of the clam, *Cumingia* [3]. In the egg of *Spisula*, the mitotic gelation occurs at approximately 44 minutes after insemination. At that time the centrifuge value rises from 5 or 6 to 11 or 12. The viscosity remains at this level until approximately 57 minutes after insemination, at which time it decreases to a centrifuge value of about 6. Preliminary experiments show that KCN (10^{-4} *M*) and sodium azide have

TABLE VI

The effect of p-dichloromercuribenzoic acid on the viscosity and cleavage of Chaetopterus *eggs.* The viscosity of control eggs at 33–36 minutes is 14.

Concentration (molar) of CMB	Viscosity at 33–36 min. after insemination	Per cent of eggs cleaved
10^{-3} *M*	8	0
10^{-4} *M*	less than 8	0
10^{-5} *M*	8	15

an effect on the protoplasm of *Spisula* eggs similar to that which they have on the protoplasm of *Chaetopterus* eggs. They inhibit the mitotic gelation and prevent cleavage. We hope to present this work in detail in a future paper.

If the gelation or clotting of protoplasm involves an oxidative process, one presumably involving –SH groups, then sulfhydryl inhibitors might perhaps prevent the completion of the gelation. But in order to act, an inhibitor must be able to enter the cell. Apparently *p*-chloromercuribenzoic acid (CMB) can enter *Chaetopterus* egg cells and inhibit mitotic gelation. Experiments with this substance are difficult, for it does not readily go into solution in sea water, and even though alkali is used to dissolve it, on bringing back the solution to the pH of sea water much of the CMB may come out of solution again. We did a number of experiments with CMB. One of these is shown in Table VI. The effect of CMB is largely reversible, though there may not be complete recovery of all eggs.

DISCUSSION

During the time between fertilization and first cleavage in the eggs of *Chaetopterus*, *Arbacia*, and *Spisula*, there are changes in the protoplasmic viscosity. In these eggs just prior to the formation of the mitotic spindle there is an increase in viscosity, the mitotic gelation, which is thought to be brought about by an enzyme which is both a protease and a clotting enzyme. This enzyme is activated by calcium ion which is released from protein and lipid in the cell cortex. Presumably the mitotic spindle forms as a result of this gelation [4]. During the time that the mitotic gelation is occurring, Rapkine [11] found a decrease in the free –SH groups in the protoplasm. Mazia [8]

has also reported that the formation of the mitotic spindle is accompanied by the conversion of –SH groups to S–S groups. Also, Hultin [6] in his studies on homogenates of sea urchin eggs has found that the clotting which occurs following addition of calcium to the homogenate is accompanied by an increase in the uptake of oxygen. These facts indicate that the gelation process is accompanied by an oxidative process. Not only is protoplasmic gelation associated with oxidation, but this is apparently also true for blood clotting in spite of reports to the contrary. Thus Rieser [12] has clearly shown that sulfhydryl reagents affect the thrombin clotting time of fibrinogen solutions. Cysteine (10^{-3} *M*) and glutathione (10^{-3} *M*) prolong the clotting time, and cysteine (10^{-2} *M*) and thioglycollic acid (10^{-3} *M*) completely inhibit the clotting process.

The inhibition of gelation in the eggs of *Chaetopterus* and *Spisula* by cyanide, azide, and *p*-chloromercuribenzoic acid is indicative of an inhibitory action by these agents on a proteolytic (gelating) enzyme system. It is well known that cyanide is a strong inhibitor of the action of trypsin [13] and of some plant proteases [14]. But if cyanide and azide inhibit a proteolytic enzyme system essential for the mitotic gelation in the eggs of *Chaetopterus* and *Spisula*, how then can one explain the fact that in the egg of *Arbacia* these same agents fail to inhibit the gelation but enhance it and inhibit its reversal? This strange behavior can be explained by the fact that cyanide acts differently on different enzymes. Although cyanide inhibits some proteolytic enzymes it is known to favor or activate others. Cyanide favors the action of cathepsin and papain [10] and activates some plant proteases [14]. Thus in the egg of *Arbacia* the proteolytic enzyme system associated with the gelating process may be favored or activated by cyanide and azide.

In general, when a living system responds to a stimulus there is an increase in the rate of metabolism. For example when a muscle contracts it consumes more oxygen and produces more heat than it does when at rest. Many biologists have regarded the increase in metabolic rate as the primary effect of the stimulus, and they have maintained that the primary effect of anesthetic agents is to prevent an increase in the rate of metabolism. However, there is a considerable amount of evidence which indicates that the primary effect of a stimulus on a living cell is the release of calcium ion from the cell cortex. The calcium ion in turn is thought to activate an enzyme which is both a protease and a clotting enzyme. The gelation involves a change of –SH groups to S–S groups and this oxidative change is thought to initiate a series of oxidative changes.

On the other hand, the primary effect of anesthetic agents is to prevent the

clotting or gelation of protoplasm, and if there is an inhibition of respiration as has been claimed in a number of cases (for example, see Ghosh and Quastel [2]), the inhibition may be due to the fact that the protoplasm has been prevented from clotting. Actually there is clear-cut evidence which shows that anesthetic agents do keep the protoplasm in a fluid state. For a complete discussion of this concept of stimulation and anesthesia consult Heilbrunn [4].

SUMMARY

1. In this paper an attempt has been made to show the relation between colloidal changes in the protoplasm and oxidative changes.

2. Potassium cyanide, at a concentration of 10^{-2} molar, prevents the mitotic gelation in *Chaetopterus* eggs, and reversibly inhibits the cleavage of these eggs.

3. In concentrations of 10^{-4} molar, potassium cyanide prevents the mitotic gelation in eggs of the clam *Spisula*, and reversibly inhibits cleavage.

4. Arbacia eggs are more sensitive to cyanide than are the eggs of *Chaetopterus* or *Spisula*. A concentration of 10^{-5} molar is sufficient to inhibit the cleavage of these eggs reversibly. However, the cyanide, although at first it keeps the protoplasm fluid, later enhances the mitotic gelation and prevents the normal reversal of this gelation.

5. The different effects of cyanide on the protoplasmic colloid of *Chaetopterus* and *Spisula* eggs on the one hand, and *Arbacia* eggs on the other, are interpreted on the basis of the fact that some proteolytic (and clotting) enzymes are inhibited by cyanide and others are accelerated by cyanide.

6. On *Chaetopterus* eggs, sodium azide acts in much the same fashion as potassium cyanide does, and at the same concentration. Thus a 10^{-2} molar solution prevents the mitotic gelation.

7. Also on *Arbacia* eggs, sodium azide acts in the same way that cyanide does, but only at concentrations about a hundred times as high.

8. The mitotic gelation is known to involve an oxidative change in which –SH groups are oxidized to S-S groups. If this oxidation is prevented by *p*-chloromercuribenzoic acid, the mitotic gelation does not occur and the eggs do not cleave.

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Experimental Cell Research **13**, *244–262 (1957)*

THE GROWTH OF SINGLE CELLS

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Received February 28, 1957

Growth is one of the fundamental properties of cells and of all living matter. The growth of cell populations is known in considerable detail, but much less is known as yet about the growth of individual cells during their "life cycle" from one division to the next. The early work on individual cell growth consisted of measurements of the increase in volume of cells with a regular geometric shape. The disadvantage of these results is that volume increase is only one of the possible measures of cell growth, and not perhaps the most interesting one. A more significant aspect of growth is the synthesis of new cellular material; but volume growth will only give a measure of this synthesis so long as the internal concentration remains constant. This constancy cannot, however, be assumed without proof, especially since it is known that many plant cells complete their growth by imbibing water and diluting themselves.

A better measure of the synthesis of new cell material would be given by the increase in total dry mass. Because of this, the work that will be described in this paper started with the intention of trying to measure the growth in dry mass during a cell life cycle. There appeared to be two ways of tackling this problem. One of them was to use mass cultures in which the cell divisions had been synchronised artificially. There has been a lot of interest in synchronous cultures in recent years and a number of different methods have been used for producing them [14]. They have the great advantage that they provide enough material for full-scale chemical analysis, but there are also attendant disadvantages. The synchrony is never perfect, so the finer details of growth curves tend to be obscured. There is also some doubt as to whether the growth of a synchronous culture is "normal" in the sense that it is the same as the growth of cells in the logarithmic phase of an ordinary unsynchronised culture. This point is discussed at the end of this paper.

The other approach to the problem of dry mass measurements was to

work with single cells. Conventional methods of determining dry mass are, of course, useless since they are far too insensitive and in any case kill the cell. Indirect methods have to be used, and, as yet, there are only two which are applicable. Reduced weight (weight in water) can be measured with a sensitive Cartesian diver balance; this is proportional to dry mass, provided the dry density of the cellular material does not change. This method has been used on *Amoebae* by Prescott [17] and has given some very interesting results. It can only be applied, however, to large cells and it is impossible to make simultaneous measurements of cell volume. The alternative method is to use an interference microscope, and measure phase retardations. The dry mass of a biological object of uniform retardation and known area can be calculated directly from its retardation to an accuracy of about ± 10 per cent [7].

The interference microscope was selected as the best of the available methods, but before it could be used to measure the dry mass of growing cells it was necessary to solve a problem of technique. A cell is an object with varying retardation, and the total dry mass can only be found by integrating the retardation over the area of the cell. This can be done by measurements on photographs but it takes a long time without special equipment. An optical method was therefore developed and tested: this is described by Mitchison, Passano and Smith [15].

Preliminary accounts of this work have already been published [15, 16].

MATERIAL

The cell that was used in this work was a fission yeast, *Schizosaccharomyces pombe* Lindner (N.C.Y.C. 132). This is a rod-shaped organism about 3.5 μ in diameter and 5 to 20 μ in length (Fig. 1). It is a convenient cell for growth measurements for a number of reasons. First, it has a regular geometric shape—a cylinder with hemispherical ends—so its volume can be calculated from its length and diameter. It grows only in length, so the increase in volume of a growing cell is directly proportional to the increase in length. Secondly, like many other microorganisms, it grows rapidly (minimum generation time of about 2 hrs at 32°C) in a sterile medium. Thirdly, it divides into two separate daughter cells of equal size after the formation of a median cell plate (which grows inwards from the outer cell wall). This type of division has a greater resemblance to that of a normal plant or animal cell than the budding of other yeasts. Fourthly, unlike many bacteria, there is no reason to suppose that there is more than one nucleus in each cell or that the nuclear and cytoplasmic division can go out of step. There is no nuclear region which can be distinguished with any certainty by ordinary nuclear stains (including Feulgen), but there is a faintly visible central vacuole or area of low refractive index which can be seen under

phase-contrast.[1] This vacuole divides and moves to the ends of the cell just before the appearance of the cell plate.

Stock cultures of the yeast were kept in the fully grown condition in 2 per cent w/v "Oxoid" Wort Broth.

METHOD

The dry mass of individual growing yeast cells was measured by means of an integration method with a Baker interference microscope. The apparatus has already been described [15], and only a brief summary will be given here. The essence of the method is to restrict the field round the object with an aperture or "box" of known area and then to measure the total retardation within this aperture due to the presence of the object. The Sénarmont compensator is adjusted until there is an equal intensity in the field with and without the presence of a quarter-wave plate. A similar adjustment is made with the object moved out of the field. The difference between the two compensator settings gives the integrated area retardation due to the object. The quarter-wave plate is motor driven, and the light intensity is measured by a photomultiplier and presented on a cathode ray tube. Under optimum conditions the instrument is sensitive to a change of about 5×10^{-14} g in the dry weight of a biological object, but in practice the sensitivity is usually less than this. A severe restriction on the use of this method is that the maximum retardation of the object must not exceed about 45° or $\lambda/8$.

There were two differences between the apparatus used for these measurements and that described in [15]. A D.C. "Pointolite" was used instead of a ribbon filament microscope lamp. This was run from the A.C. mains through a rectifier and smoothing circuit, and gave somewhat more light. Secondly, the growing yeasts were kept at a temperature of 25°C by mounting the slide in a small warm stage placed on top of the microscope stage.

One of the major problems in using this method on living cells is to find a satisfactory way of mounting them. There are three difficulties that have to be overcome in the case of the fission yeast. First, the maximum retardation of the cells in water is about 80° while the maximum retardation that is permissible for the apparatus is 45°. It is therefore necessary to reduce the retardation of the cells by mounting them in a medium of higher refractive index than water. Since the concentration of solids in the cells is 30–40 per cent (g/100 ml) the concentration of solids in the mounting medium has to be 15–20 per cent. In addition, the material added to the medium should neither penetrate the cells nor prevent their growth. Secondly, it is important to prevent the cells moving about either by Brownian movement or by slow drift due to currents in a fluid medium. The main reason for this lies in the variations in the thickness of microscope slides and cover slips. The method of measuring the cell retardation assumes that these thicknesses remain constant. In fact they vary, but provided the cell stays in the same place this only produces a small constant error in the absolute value of the dry mass. If, however, the cell is moving about over the slide the error will change from point to point and will produce irregularities in the growth curve. Thirdly, the high power shearing objective (× 100, water immersion

[1] Leupold [12] has successfully stained this central area by the Feulgen technique.

with coverslip) of the Baker interference microscope has a very short working distance. If the cells settle on the slide, the thickness of the medium above them has to be so small that it affects their growth. Apart from working with an inverted microscope, the only solution to this difficulty is either to stick the cells to the underside of the coverslip or to hold them in this position.

After a number of trials, it was found that the best way of satisfying these requirements was to mount the cells just below the coverslip in a gel with a nominal concentration of 17.5 per cent gelatin and 2 per cent Wort Broth. This gel prevented the cells from moving and also had a refractive index sufficiently high to reduce the maximum retardation of the yeast cells to 45° or less. Growth tests showed that the presence of the gelatin did not affect either the rate of growth or the final concentration of the cells in a fully grown culture, as compared with a plain Wort Broth medium. There is also no evidence that the presence of a gelated medium affected the optical performance of the microscope. The medium round a growing cell showed no visible signs of strain and had the same retardation as the rest of the medium in the vicinity.

The gelatin medium was prepared in batches by dissolving 35 g of bacteriological gelatin (Gurr) and 4 g of Wort Broth (Oxoid) in 150 ml of hot distilled water. The solution was cooled to 55°C, and made up to 200 ml after the addition of 10 ml of 20 per cent egg albumen. It was heated in a boiling water bath for two hours, by which time the albumen had precipitated and cleared the solution. The albumen precipitate was filtered off in a hot oven (70°C) and the medium put into McCartney bottles in 5 ml lots. Finally, these were lightly autoclaved (5 mins at 10 lbs/in^2). This medium gelled at 27.5–28°C, though the gelling point lowered with longer autoclaving. The dry weight of a sample from each batch was measured; it varied between 0.16 and 0.17 g/ml in different batches. The χ value of the medium was found to be 0.184 by measurement with a refractometer (Na_D).

The experimental procedure started at 6 p.m. with the inoculation of 5 ml of liquid gelatin medium at 32°C with one loop of a fully grown culture. This was grown overnight at 28°C and at about 10 a.m. the next morning it was fully liquefied by warming to 32°C. A small drop of the shaken culture was placed on a flamed slide and covered by a flamed No. 0 coverslip supported on either side by strips of No. 3 coverslip. About half the space between the slide and coverslip was filled with the medium; the rest with air. The slide was then ringed with vaseline and inverted, coverslip downwards, in an oven at 32°C. It was left in the oven for 20 minutes by which time most of the cells had fallen through the liquid medium and were resting just above the coverslip. The slide was then removed from the oven, left at room temperature (18–20°C) for 10 minutes in the same inverted position to allow the medium to gelate, and finally placed in the normal position (coverslip upwards) in the warm stage on the microscope at 25°C. The effect of this procedure was to leave the cells held in a gelated medium just below the coverslip and with a substantial thickness of the medium (about 250 μ) below them.

The slide was left in position on the microscope for 1–1 ½ hrs to allow the possible effects of the temperature shocks on mounting to wear off and normal growth to recommence. A suitable cell was then selected; it had to be single, not too near division, accurately horizontal, with a uniform background round it, and lying in such a position that neither it nor the reference beam was near other cells. When

found, the cell was positioned in the centre of the aperture; this aperture being set to an area about 5 to 10 times the area of the cell. Measurements were made by taking 5 compensator readings with the cell in position, followed by another 5 readings on an empty part of the background immediately adjacent to the cell. The retardation of the cell was twice the difference between the means of the two sets of readings. The photomultiplier assembly was then replaced by a camera and a photograph was taken. This procedure was repeated every 7½ mins until the end of the experiment (2–8 hrs). The × 100 water-immersion shearing objective was used in all the main experiments, together with a condenser N.A. of 0.3.

In some cases, the cells divided in line and the experiment only finished when they grew out of the aperture. These gave satisfactory runs of a length determined by the dimensions of the aperture. A long run was possible with a large aperture, but at the same time this reduced the accuracy of the readings. The best compromise seemed to be to have a box which could just accomodate four small cells in line. This would allow for one complete cell life cycle. However, in many cases something went wrong and the experiment had to be stopped before the second division. The most usual cause of trouble was an irregular division in which the two daughter cells did not stay in line. They either arranged themselves in a V, in which case they grew out of the aperture soon after division; or they ceased to lie horizontal and so prevented a good photograph being taken.

The dry mass of the cell (m) was calculated from the following formula:

$$m = \frac{x A \lambda}{360 \chi} + \frac{\chi' c V}{\chi},$$ [1]

where x is the retardation of the cell in degrees; A is the area of the aperture of the box; λ is the wavelength of the light used (0.54 μ); c is the concentration of solids in the medium in g/ml; V is the volume of the cell; χ' (= 0.184) is 100 × the specific refractive increment of the medium; and χ is the same for the cell. χ was assumed to be 0.18, a reasonable value for a biological object [7]; but, even if this is not correct, it is only likely to produce a small error in the absolute values of dry mass and it will not affect the shape of the growth curves. The shape of the curves will only be affected if the χ value changes during the life cycle due to large alterations in the chemical composition of the cell.

The volume of the cell was calculated by the following formula, from dimensions measured on the photographs:

$$V = \pi D^2 (L - D/3)/4,$$

[1] The similar equation (d) in [15] should have a plus rather than a minus sign on the right-hand side.

where D is the diameter, and L the total length of the cell. This is the formula for a cylinder with hemispherical ends.

The total percentage concentration (g/100 ml) of the cell was taken as $100\ m/V$.

At the time when the slide was made up from the culture, the concentration of cells in the culture was $1–2 \times 10^6$ cells/ml. The final concentration of the cells in a fully grown culture in the gelatin medium (5 ml in a McCartney bottle) was about 40×10^6 cells/ml. Therefore, at the time of mounting, the cells must have been in the middle of the logarithmic phase of growth. A concentration of about 10^6 cells/ml is the optimum for the method of mounting and measurement. If the cells are more dilute, it becomes very difficult to find a suitable one anywhere on the slide. If they are more concentrated, it is difficult to find one sufficiently isolated from its neighbours.

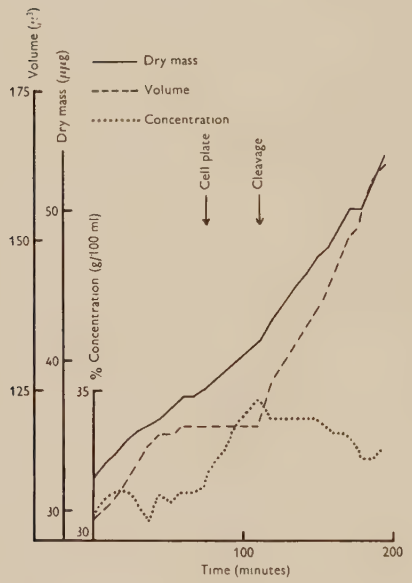
A strongly birefringent object will give spurious results with a Baker interference microscope, and the retardation will vary with its orientation. The cell wall of this yeast is faintly birefringent, so a test was carried out to see whether the retardation of a yeast cell varied with its orientation. It did not, so the effect of the birefringence can be assumed to be negligible.

RESULTS

At the beginning of this work, a series of ten pilot experiments were done, a $\times 40$ objective being used and measurements being taken every fifteen minutes. The results from these experiments have not been included in the data given below, but they showed no obvious differences from what was found later. The main experiments were done with a $\times 100$ objective and more frequent readings (every $7\frac{1}{2}$ mins). 17 of these experiments gave runs which were long enough for an adequate analysis (3–8 hrs). In the other cases either the cell failed to grow or it moved out of focus after a short time.

The results from three experiments are shown in Figs. 2, 3 and 4 as graphs of dry mass, volume and concentration plotted against time. In Figs. 2 and 3, a cell was followed through its cleavage into two daughter cells and then well into interphase. The run shown in Fig. 4 was unusual because the cell could be followed through two cleavages, and growth measurements were possible on the four grand-daughter cells. All these runs had eventually to be stopped because the cells grew out of the aperture. The larger fluctuations of the dry mass curve in Fig. 3 were due to the use of a bigger aperture than in the other two experiments.

The main features of all the growth curves can be seen in Figs. 2, 3 and 4. The volume curve shows a slight but definite upwards curvature throughout most of interphase, indicating a slow increase in the rate of growth of volume. About an hour before cleavage, however, the curve flattens off abruptly and there is a "constant volume stage" during which there is no volume growth (this feature is obscured in the second cleavage in Fig. 4 by the slight asyn-



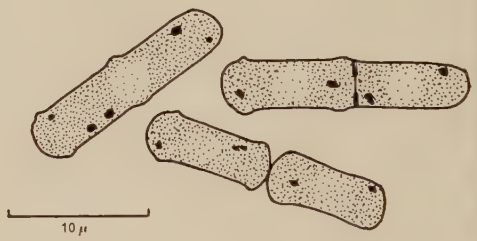


Fig. 1.—*(Right.) Schizosaccharomyces pombe* in phase contrast. Central vacuoles, lipid granules, and bumps due to division scars are shown.
Fig. 2.—*(Left.)* Growth curves. One cell through one division to two cells.

chrony of division in the two cells). The cell plate appears about one third of the way through this stage – 40 mins before cleavage. The central vacuole divides in the early part of the constant volume stage before the cell plate has appeared and this may perhaps represent "mitosis" or the division of the nuclear material. The final stage of cleavage takes place quite rapidly in a matter of minutes: the cell plate splits down the middle and the two new cell ends round off. Growth in volume begins immediately after cleavage, but the rate, even for the two cells combined, is less than it was for the single cell just before the constant volume stage.

The dry mass growth curve differs from the volume growth curve in two main ways. First, there is no constant stage before cleavage. During the constant volume stage, the dry mass continues to increase. Secondly, the mass growth is *linear* for nearly the whole life cycle of a cell. The growth

rate remains constant up to a point near cleavage when it changes fairly rapidly to the new and higher rate for the next generation.

The curve for total cell concentration is derived from the mass and volume results. During the constant volume stage it rises sharply to reach a maximum at cleavage. It then falls for the rest of the life cycle and reaches a minimum

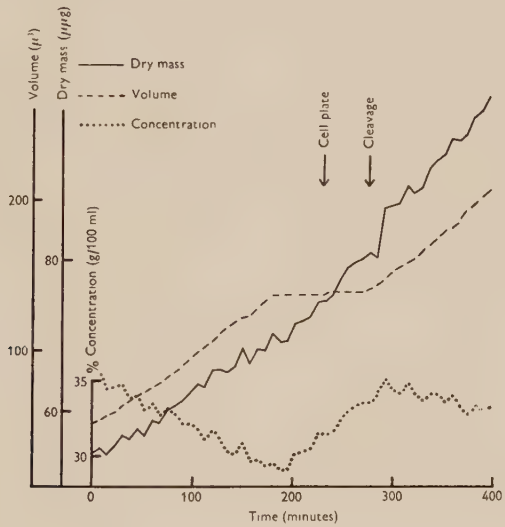


Fig. 3.—Growth curves. One cell through one division to two cells.

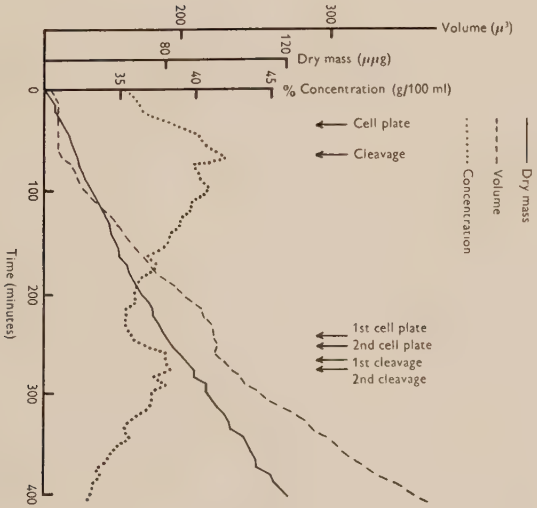


Fig. 4.—Growth curves. One cell through two divisions to four cells.

at the start of the constant volume stage. In Fig. 4 there is an overall fall in concentration in addition to these cyclical variations. This occurred in a number of experimental runs.

Although nearly all of the experimental curves showed the features that have just been mentioned, it was necessary to do a statistical analysis of the results both to summarise them and also to prove the important point of a linear growth rate. Before describing this analysis, however, five exceptional curves must be considered. In three of them, there was an initial upwards curvature of both the mass and volume curves for the first 50–100 minutes. Thereafter, the mass and volume curves behaved normally, with the mass growth being linear during the rest of the first interphase and during the second interphase. These results were almost certainly due to the observations being started too soon after the temperature shock on mounting; the cells had not fully recovered. The initial portions of these curves were not, therefore, included in the analysis. In the fourth case, the mass curve showed a most unusual feature. About half way through the two cell stage, the growth rate, which had been constant, suddenly increased three times and continued at the new rate. The volume growth curve, however, showed no discontinuity and behaved normally. I cannot explain this result and have decided to omit it from the analysis because it lies far beyond the limits of variation of the other mass curves and is completely different in character. In the fifth case, the mass curve was not linear in the two cell stage but showed a definite but small downward curvature. This result has been included in the analysis and will be considered below.

For the purpose of analysis, each experimental curve of mass and of volume was divided into one or more sections corresponding to the generations. The first section, for the one cell stage, ran from the beginning of the experiment to the start of the constant volume stage before the first division. The second section, for the two cell stage, ran from the completion of the first cleavage to either the end of the experiment or to the start of the constant volume stage of the second division. The constant volume stage was therefore omitted from the analysis of both the volume and the mass curves. Since the beginning of this stage was uncertain in a number of cases (e.g. the second division in Fig. 4), it was convenient in practice to make a fixed rule that the section should stop at a point where the time interval between it and the cleavage was equal to twice the interval between the appearance of the cell plate and cleavage. In most of the curves this point was slightly earlier than the beginning of the constant volume stage.

A quadratic curve (of the form $y = A + Bx + Cx^2$) was fitted to each section

of the mass and volume curves, the method of orthogonal polynomials being used [9]. In view of the small and regular curvature it was unnecessary to fit a polynomial of higher degree or an exponential curve. A summary of the data from these fitted curves and from the experimental curves is given in Table I. The slope of the fitted curves is given by the linear coefficient B for the mass curves (where, as will be shown, the quadratic term is not significant) and by the first differential $B-2Cx$ for the volume curves. It should be noted that the slope of the mass curves only increases by an average factor of 1.57 at the first division. If the two daughter cells were growing in mass at the same rate as their mother cell, the factor would be 2. The curvature of the fitted curves is given by the quadratic coefficient C. The other data in Table I are taken from the experimental curves.

It was important to establish that the rate of growth of mass was constant for each generation. Three tests for linearity were therefore applied to the sections of the mass curves. First, each section was taken separately and the significance of the quadratic variance was tested against the residual variance. In 26 out of 27 sections the quadratic term was not significant for $P = 0.05$, so the sections fitted a line better than a quadratic curve. In one case, which has been mentioned above, a quadratic curve gave a better fit. Secondly, a more sensitive test for curvature (irrespective of sign) was made by finding the χ^2 value for the combined probabilities from all the sections [8]. This gave a result similar to the individual tests: with the 26 linear sections, there was no significant curvature ($P = 0.22$); but if the curved section was included, there was a significant curvature ($P = 0.0045$). Thirdly, a test of significance was made on the sum of the quadratic coefficients weighted by the reciprocals of their residual variances. This was not significant for all 27 sections ($P = 0.34$), showing that there was no significant tendency of curvature in either direction (i.e. taking account of sign). The conclusion from these tests is that the growth in mass is linear for each generation. The one curved section made the combined probability test show curvature but it seems reasonable to regard this as an indication of the sensitivity of the test and not as a proof of the general curvature of the mass growth.

One remaining uncertainty about the mass growth is when the rate for one generation changes to the rate for the succeeding generation. In the experimental curves, it appears to occur during the constant volume stage and in some cases the change seems to be rapid (Fig. 2); but it tends to be obscured by the variability of the readings and the fact that the increase in rate is not very large. Another method of approach is to calculate the meeting point of the two fitted lines for the sections of the mass curve. This also indicates

TABLE I

Data from growth curves.

Schizosaccharomyces pombe. Wort broth gelatin. 25°C.

	Units	Mean	Standard deviation (±)	*n*
		Dry mass		
No. of points in each section of growth curve		15.1	5.4	27
Slope. 1 cell	$\mu\mu$g/min	0.092	0.015	14
Slope. 2 cells	$\mu\mu$g/min	0.134	0.039	12
Slope. 4 cells	$\mu\mu$g/min	0.246	—	1
Increase of slope. 2 cells/1 cell		1.57	0.72	9
Increase of slope. 4 cells/2 cells		1.52	—	1
Curvature. Quadratic coefficient	10^{-3} $\mu\mu$g/min^2	0.05	1.95	27
Mass of each cell at cleavage. 1 cell	$\mu\mu$g	54.9	12.7	17
Mass of each cell at cleavage. 2 cells	$\mu\mu$g	43.3	6.3	5
Mass increase during constant volume stage	$\mu\mu$g	5.8	2.0	17
		Volume		
No. of points in each section of growth curve		15.8	6.2	30
Slope. Finish of 1 cell stage	μ^3/min	0.408	0.109	14
Slope. Start of 2 cell stage	μ^3/min	0.373	0.102	15
Slope. Finish of 2 cell stage	μ^3/min	0.618	0.232	4
Slope. Start of 4 cell stage	μ^3/min	0.867	—	1
Increase of slope:				
Start 2 cells/Finish 1 cell		0.906	0.194	12
Finish 2 cells/Start 2 cells		1.724	0.708	4
Start 4 cells/Finish 2 cells		1.133	—	1
Curvature. Quadratic coefficient	10^{-3} μ^3/min^2	1.343	1.613	30
Vol. of each cell at cleavage. 1 cell	μ^3	148.5	34.3	17
Vol. of each cell at cleavage. 2 cells	μ^3	126.8	29.1	5
		Concentration and Time		
Max. concentration of cells. At cleavage	g/100 ml	37.2	4.5	17
Min. concentration of cells. At start of constant volume stage	g/100 ml	33.1	3.8	17
Generation time; between successive appearances of cell plate	mins	258	46	8
Duration of constant volume stage	mins	65	16	17
Time between first appearance of cell plate and cleavage	mins	39	9	21

that the change in rate happens during the constant volume stage, but the results are rather variable.

Significance tests were also done on the sections of the volume growth curve. Individual tests showed that 20 out of 30 sections gave a better fit to a quadratic curve rather than a line. A combined probability test gave a highly significant curvature, and it could be seen by inspection that the curvature was positive or upwards (there were no negative curvatures). The conclusion, therefore, is that each section of the volume curves shows a slight but definite upwards curvature. The rate of growth of volume increases through most of a cell life cycle.

During the constant volume stage, the cell dry mass increases on an average by 5.8 $\mu\mu$g. It is interesting to know how much of this increase is due to the production of new material in the cytoplasm. An experiment was therefore done in collaboration with Dr. P. D. Mitchell to determine the dry thickness of the cell wall. A suspension of empty cell walls was obtained by treatment of the cells in a Mickle disintegrator. The dry weight of these cell walls accounted for 21.7 per cent of the total dry weight of the cells. This figure, together with an assumed cell wall density of 1.3 g/ml and measurements of cell numbers and mean cell size, gave a thickness of 450 Å for the dry cell wall. Measurements with an interference microscope and half-shade eyepiece of the retardation through the empty cell walls gave a dry thickness of 860 Å. The interference method probably gave an overestimate of the thickness since there appeared to be some of the cell contents left inside the sacks of cell wall. The dry weight method may have given an overestimate for the same reason, but it may also have erred in the other direction since the smaller fragments of cell wall are likely to have been lost on washing and centrifuging. On balance, it seems reasonable to take a rough figure of 600 Å for the dry thickness of the cell wall. If it is assumed that the fully-formed cell plate is a double thickness of cell wall with a diameter of 3.43 μ (mean of 17 measurements), and that the density of the wall material is 1.3 g/ml, then the dry volume of the cell plate is 1.1 μ^3 and the dry mass is 1.4 $\mu\mu$g. The laying down of the cell plate, therefore, only accounts for $1.4/5.8 = 24$ per cent of the increase in dry mass during the constant volume stage.

A similar calculation shows that cell wall growth accounts for nearly the same proportion of the total mass growth just before the constant volume stage. At this point, the mean rate of volume growth is 0.408 μ^3/min. Assuming, as before, a wall thickness of 600 Å and a density of 1.3 g/ml for its material, the rate of increase of volume of the dry wall is 0.0285 μ^3/min and of mass is 0.0371 $\mu\mu$g/min. But the rate of increase of dry mass of the

whole cell is 0.092 $\mu\mu$g/min, so the cell wall growth accounts for 0.0371/0.092 = 25 per cent of the total mass growth.

It is possible to calculate the concentration of the cytoplasm by subtracting the contribution to the total dry mass made by the cell wall and cell plate. At cleavage, the mean surface area is 186 μ^2 (derived from the volume 148.5 μ^3 and the diameter 3.43 μ). Assuming a wall thickness of 600 Å and a density of 1.3, this gives the dry volume of the wall as 11.13 μ^3 and its dry mass as 14.5 $\mu\mu$g. The total dry mass of the cell at cleavage is 54.9 $\mu\mu$g, so the dry mass of the cytoplasm is 54.9–14.5–1.4 = 39.0 $\mu\mu$g. In a volume of 148.5 μ^3, this gives a concentration for the cytoplasm of 26.3 per cent (g/100 ml). A similar calculation for the cell at the start of the constant volume stage gives a concentration of 23.3 per cent.

After division, an annular scar is left at the end of each of the daughter cells which marks the edge of the preceding cell plate. This "division scar" is similar to the bud scars found on budding yeasts [3]. In isolated cell walls, these scars are visible both under phase contrast and under the electron microscope as annular thickenings near the ends of the cells. They can also be seen in intact cells as small bumps in the cell profile (Fig. 1). By taking these bumps as reference points, it is possible to determine whether a cell grows at one end, both ends, or in the middle. It was found that most, but not all, cells grew at one end. A few grew at both ends, and none in the middle. After division a daughter cell has a "new end" which has just been formed from the cell plate and an "old end". It was found that nearly all cells grew at the old end.

DISCUSSION

Dry mass growth.—The most interesting result of these experiments is the finding of linear growth in mass for each generation. A cell can be regarded as a factory producing new cellular material. This factory doubles in size during its life cycle, so one might well expect its rate of production to double as well, provided that there was an adequate supply of raw materials. This would give a smooth exponential curve of growth, similar to the growth of cell populations, which would show no discontinuity at division. But this does not happen: instead, it appears that once a cell has been formed it maintains a constant rate of synthesis of new material until the end of its life cycle, even though it has doubled in size. It is, of course, tempting to speculate how this rate of synthesis is controlled. If the presence of a nucleus is assumed in this yeast, this might be the controlling factor since a nucleus in other cells remains as a constant morphological unit through nearly all

the life cycle. The difficulty of this idea is that one would expect an interruption in growth prior to division when the nucleus was occupied in mitosis or its equivalent. Although the volume growth does show an interruption, there is no sign of it in the mass growth which is a much better measure of synthesis. A more likely hypothesis is that the rate of growth is controlled by the number of active microsomal particles; these being structures which are thought to be closely concerned with protein synthesis in other cells. If so, the number of particles should remain constant until just before cleavage when they would normally double their numbers and partition themselves between the two daughter cells. It should be possible to test this hypothesis by seeing whether growing cells contained a constant number of microsomal particles.

The argument above rests on two important assumptions. First, there is the tacit assumption that the rate of production of new cellular material is the same as the rate of growth in dry mass. Phrased in another way, this assumes that the relative proportions of proteins, free amino acids, carbohydrates, etc. remain the same. It might be, however, that at one stage of the life cycle the cell contains a high proportion of "undigested" food material, like a man after a meal. The only virtue of measuring dry mass rather than volume is that it eliminates the role of water in growth. This assumption of constant composition is a difficult one to justify because of the lack of information. Prescott [17], however, found that the growth in protein content of *Amoebae* was very similar to the growth in reduced weight. This indicates that the proportion of protein in a growing cell does not change. Further evidence on this point is likely to come from the analysis of synchronous cultures, and we are attempting to do this at the present time with *Schizosaccharomyces pombe* in this laboratory.

The second assumption is that the linear growth in mass found in the experiments also occurs in normal logarithmic growth where it is assumed that each generation grows at the same rate as the preceding one. In the experiments, however, the second generation grew at a slower rate than the first—by a factor of $\frac{1}{2} \times 1.57 = 0.78$ in the mass rate. This was presumably due to unfavourable conditions on the microscope slide such as deficiencies of oxygen and food or accumulation of CO_2, alcohol and waste products. It might therefore be argued that the linear growth rate was due to these conditions and that normally the growth in mass followed an upward curve. There are two objections to this argument. The first comes from the work of Mr. M. J. W. Faed in this laboratory (as yet unpublished). He has developed a method of observing this fission yeast under the microscope in a

chamber through which fresh medium is circulating. Under these conditions, there is no falling off of the growth rate at division. It is not possible with this method to use the interference microscope to measure dry mass, but volume measurements can be made from films taken with an ordinary microscope. He has found that the volume growth curves have a very similar shape to those described in this paper. Provided there is the same relation between mass and volume growth in this method as there is under the interference microscope, this implies that the growth in dry mass during normal logarithmic growth is also linear for each generation. Secondly, if the effect of the unfavourable conditions under the interference microscope had been to flatten what was normally an upward curve for mass growth, it seems very unlikely that a straight line would have been produced in nearly every case. These two points seem to me to be valid objections to the hypothesis that the mass growth is curved under normal conditions, but even if they are not accepted, it is clear that normal mass growth cannot follow a smooth exponential curve and must show discontinuities. The reason for this is that the experimental curves do in fact show discontinuities at division and these cannot have been produced by unfavourable conditions alone.

If the idea of linear growth under normal conditions is accepted, an interesting deduction can be made from the experimental results. Under unfavourable conditions, the mass growth is still linear, but it slows down for each succeeding generation. This suggests that for the purpose of growth, a cell only takes notice of mildly adverse conditions at division when the new growth rate for the succeeding generation is fixed. The conditions, however, must affect the cell before division so it is possible that they act by slowing down the production of some precursor of the synthetic machinery, perhaps RNA, and so prevent the machinery doubling at division. One way of testing this suggestion would be to use Faed's technique and see whether low concentrations of inhibitors or dilution of the growth medium reduce the growth rate of a cell at once, or only at the next division. This suggested delay in the reduction of the growth rate would only, of course, happen in a mildly adverse environment. Under drastic conditions, such as a total absence of food or a lethal concentration of an inhibitor, growth would stop at once.

The mass growth rate does not appear to be determined by the surface available for the intake of food. If it was, the mass growth should follow a curve which was nearly exponential. Nor can it be assumed that food intake only takes place at the actively growing end of the cell, since in this case there should be no mass growth during the constant volume stage.

Before leaving the problem of mass growth, it is worth making a comment about logarithmic growth. It has been assumed so far that the growth rate

of individual cells remains the same during "normal" growth when the cell-numbers are increasing logarithmically. But in many micro-organisms, including this yeast, the mean cell size decreases during most of the logarithmic phase of growth. This means that the individual cell growth rate is diminishing in succeeding generations. Although this decrease is not as large as the factor of 0.78 found in the experiments, it implies that the conditions under the interference microscope are not as abnormal as might be supposed.

Volume growth, cell wall growth and concentration changes.—The curves of volume growth are sharply divided into two stages. In the first one, which lasts for 75 per cent of the generation time, the volume increases at a rate which is slightly faster than the mass growth and which also accelerates. In the experimental curves the rate of growth was 1.7 times faster at the end of this stage of growth than it was at the beginning. The rate of deposition of cell wall must also behave in the same way. Since the volume growth is faster than the dry mass growth, the total cell concentration falls from 37 to 33 per cent and the concentration of the cytoplasm falls from 26 to 23 per cent. In the second stage, the constant volume stage which precedes division, there is no change in volume. During this stage, the point of deposition of new cell wall moves from one end of the cell to the centre and the cell plate is laid down starting from the outside and moving inwards. The overall rate of deposition of the cell wall is the same as it was at the end of the previous stage of growth since both of them account for about 25 per cent of the total rate of mass increase and this latter rate remains constant. There is a lag of about 25 minutes between the start of the constant volume stage and the first appearance of the cell plate. This may imply a delay before the cell plate deposition starts, but, if so, it will probably be less than 25 minutes since the cell plate will not be visible under the microscope during its early stages. Meanwhile the dry mass has continued to increase, so the cell concentration rises from 33 to 37 per cent and the cytoplasmic concentration from 23 to 26 per cent. Finally, cleavage takes place, the two daughter cells start their growth in volume, and the cycle begins again. Surprisingly, the point of deposition of the new cell wall in each daughter cell moves back from the new end formed from the cell plate to the old end of the cell.

It is difficult to give any adequate explanation of the existence of the most interesting part of the volume growth curve—the constant volume stage. It may be, however, that there is an upper limit on the rate of synthesis of the cell wall and, if the cell is engaged in making a cell plate, it cannot also lay down cell wall at its end. There is also a possible reason why the existence of this stage may be advantageous. The cell concentration rises to a maximum at cleavage and the internal hydrostatic pressure will probably rise with it.

This increase of pressure may well give mechanical help in the final stage of cleavage when the cell plate is split down the middle and the two new cell ends round off.

Finally, it is worth emphasising one point about the growth of the cell wall. During most of the life cycle, the cell wall is being synthesised at an increasing rate. Also, during the constant volume stage it continues to be laid down (in the form of the cell plate), at a rate which seems to be about the same as it was just before this stage. This suggests that the growth of the cell wall is the only one of the growth curves which have been considered that follows an exponential curve without any great discontinuity at division. If so, cell wall synthesis, although part of the total synthesis of new material, must be controlled by a different mechanism. This is an interesting point but, unfortunately, it is impossible to be certain about it since the figures for the rate of deposition of the cell plate are only approximate.

Other work.—Much work was done in the earlier years of this century on the growth in volume of single microorganisms. This has been reviewed by Adolph [1], and, in the case of Protozoa, by Richards [18]. Richards concludes that the growth curves are sigmoid, but there is a considerable variation in the results and some of the older work does not appear to be very reliable.

The only single cell measurements on *Schizosaccharomyces pombe* were made by Knaysi [10]. He made the discovery that volume growth stopped during division, but his readings were too sparse (5–7 per generation) to allow for an accurate plot of the volume growth curve. He also measured the growth of *Bacillus cereus* and *Streptococcus faecalis* [11] and found that whereas the former showed more or less continuous growth, the latter showed a slackening of growth during division, like the fission yeast.

Bayne-Jones and Adolph [4] made a series of careful measurements on the volume growth of the budding yeast *Saccharomyces cerevisiae*. At 37°C, they found that the rate of growth increased for the first 20 mins, stayed constant for the next 50 mins, and then declined to zero. Similar results were found by Lindegren and Haddad [13]. The linear growth which occupies most of the generation time is an interesting feature and, like the linear mass growth in the fission yeast, argues against an autocatalytic theory of growth. However, Adolph and Bayne-Jones [2] found a different picture in *Bacillus megaterium*. Here the volume growth followed an upward exponential curve and it was the rate of *relative* volume growth which remained constant.

Prescott [17] has recently published an important paper on the growth of *Amoeba proteus*. As well as measuring cell and nuclear volume, and protein content, he also used a diver balance to measure reduced weight. His results

show that reduced weight, cell volume and protein content all follow a very similar growth curve in which the rate declines continuously throughout the life cycle, finishing just before division with no growth. These results are of course very different from those given in this paper for the fission yeast. Many of the differences may be due to the type of cell used. There is no inherent reason why a small plant should grow in the same way as a large animal cell. Also, the methods of feeding differed. The yeasts were absorbing food molecules from a soluble medium, while the *Amoebae* were catching *Tetrahymena*. This might perhaps explain the cessation of growth before division in the *Amoebae*. They are known to round up at division [6] and this would presumably prevent them catching their food. There is, however, a discrepancy between Prescott's results and those of Chalkley [5] who found with the same animal that the rate of growth of volume *increased* during at any rate the earlier part of a cell life cycle.

Zeuthen [19] used a diver technique to measure the respiration of the early synchronous stages of single-cell cultures of *Tetrahymena pyriformis*. He found that there was a linear rate of increase of respiration between divisions. Arguing, somewhat indirectly, that this implied a constant rate of synthesis, he suggested that synthesis was controlled by cellular units which doubled at division. This is, of course, the same hypothesis as the one in this paper, and put forward for the same reason. One difference between Zeuthen's results and the mass growth of the fission yeast is that there is a plateau in the respiration curves just prior to division when the rate of respiration remains constant. An interesting comment on synchronous cultures induced by temperature shocks is that Zeuthen and Scherbaum [20] found that the respiration of these cultures was not the same as that found in the more normal single-cell cultures. In the synchronous cultures it was not linear between divisions. This suggests that results on the growth of synchronous cultures are not necessarily applicable to normal unsynchronised growth. Another indication of the abnormality of synchronous cultures is the finding by Zeuthen and Scherbaum that the cells are larger and divide faster than those in an ordinary culture.

SUMMARY

1. Measurements were made of the growth in dry mass and volume of single cells of the fission yeast *Schizosaccharomyces pombe*. Dry mass was measured by an integration method with an interference microscope. Volume was measured from photographs. The yeast cells were grown in a gelled medium containing 17.5 per cent gelatin in order to reduce the phase retardation of the cells and to keep them still.

2. The growth in dry mass was linear for each generation. Once a cell has been formed it maintains a constant mass growth rate until just before cleavage when the rate changes to that for the next generation. This suggests that synthesis is controlled by centres or particles, perhaps microsomal, which remain constant until division, when they normally double and partition themselves between two daughter cells. It is also suggested that, under mildly adverse conditions, the growth rate only changes at division, possibly because an insufficient quantity of a precursor has been made during the preceding generation.

3. The growth in volume is different from the growth in dry mass. For the first three-quarters of a cell cycle, the volume growth rate slowly increases. This is followed by a constant volume stage in which there is no volume growth. The cell plate appears one-third of the way through this stage.

4. The cell concentration, derived from the mass and volume figures, fluctuates cyclically, with a minimum at the start of the constant volume stage and a maximum at cleavage.

I should like to express my gratitude to Professor M. M. Swann and Dr. L. M. Passano for their collaboration in the early stages of this work; to Drs. L. J. Hale, D. Michie and D. N. Lawley for their help with the statistics; and to Miss Y. Orr for technical assistance.

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Experimental Cell Research **13**, *263–275 (1957)*

EXPERIMENTS ON THE FUNDAMENTAL EFFECTS OF FREEZE-SUBSTITUTION

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Received March 2, 1957

The term "freeze-substitution" was introduced by Simpson [19] to describe a technique for the dehydration of tissue at low temperatures. In reality, two separate stages are involved. In the first, as also in freeze-drying, the tissue is "quenched", or frozen, as quickly and to as low a temperature as possible. In the second stage, the tissue is transferred rapidly to a bath of cold organic solvent in which the ice slowly dissolves. In freeze-drying, of course, the ice is sublimed off at low temperature *in vacuo*, and a high vacuum system, often troublesome to maintain and costly to install, is therefore indispensable.

Following Simpson's [19] original work in this field, other experiments have been described. Lison [16] applied the principle to a study of liver glycogen; Russell *et al.* [18] to the preparation of radioautographs of plant tissue. Winteringham *et al.* [22] used freeze-substitution for preserving water-soluble tracer substances intact in sections of larvae. Blank *et al.* [3] described the use of propylene glycol for cold dehydration and carbowax for embedding, whilst Taft [21] employed *b*-butanol as ice solvent. Deitch and Godman [4] applied freeze-substitution to the fixation of tissue cultures. Freed [7] carried out an extensive investigation on the comparative merits of freeze-substitution and freeze-drying.

While there seems to be general agreement that the histological and cytological results of freeze-substitution are excellent, there is a division of opinion as to the fundamental effects of the cold solvent upon the tissue. Simpson [19] himself believed that it led to a fixation essentially chemical in nature; that proteins are precipitated in a denatured state, and react to stains in a fashion characteristic of chemically-fixed tissues. Bell [1] also suggested that the fixation should be regarded as chemical. On the other hand, Freed [7] considered that denaturation by the substituting medium was reduced compared with effects on fresh tissue, and that, in other respects, the properties of freeze-substituted resembled those of frozen-dried tissue. The question of how freeze-substitution affects tissue is an important one, because

the method is easier and cheaper than freeze-drying and perhaps more consistent in its results. Provided its limitations can be defined, it would seem, as Lison [15] has pointed out, to have much to recommend it in critical cytology and cytochemistry. The experiments described below provide some fresh evidence on this problem.

MATERIALS AND METHODS

Hitherto, refrigeration of the ice solvent has been carried out with freezing mixtures of various sorts. Temperatures around $\pm 70°C$ can be obtained without much difficulty with ethanol/CO_2 mixtures, but prolonged dehydration times are necessary. A temperature around $-40°C$, entailing shorter dehydration, can be obtained with ethyl oxalate/CO_2 mixtures [6]; however, such mixtures become increasingly troublesome to manage as time elapses and it is difficult to extend their life satisfactorily beyond 48 hr. An apparatus employing mechanical refrigeration was therefore constructed for the experiments described below.

This consists of a $\frac{1}{4}$ h.p. sealed refrigeration unit housed in a wheeled cabinet which is fitted at the top with a rectangular copper tank measuring 50 × 45 cm. The tank is covered by an insulated lift-off lid, and its walls are traversed by cooling coils through which the refrigerant ("Arcton 5") is pumped. It is filled with industrial alcohol. A large Pyrex beaker containing the ice solvent stands on the floor of the tank, surrounded by the cooled spirit; the beaker is fitted with an internal sliding sleeve, which can be submerged to any chosen depth in the solvent. It carries metallic gauze tissue baskets. Temperature is controlled thermostatically to within 2°C.

The main advantages in comparison with orthodox freezing mixtures are, first, that the apparatus runs continuously for days or weeks without attention. Second, since samples of tissue can be inserted into the individual baskets and recovered again at any time, the apparatus can be used by several workers independently. Third, the working temperature can be regulated to any chosen point simply by turning the thermostat knob.

As even minor variations in technique may affect the final results adversely, the method employed will be described in full.

Quenching.—A silica dish containing isopentane (B.D.H.) was lowered cautiously into a Thermos bowl containing liquid nitrogen. Small pieces of tissue (rat) were meantime prepared; these were approximately 1–1.5 mm^3. As the stirred isopentane began to thicken, three or four tissue pieces were loaded on to the tip of a narrow (1–2 mm) strip of thin aluminium foil. When the isopentane had reached the optimal state of cooling, viscous, and on the point of solidification at around $-190°C$, the foil bearing the fragments was quickly plunged beneath its surface. After $\frac{1}{2}$ min. it was rapidly withdrawn, the samples general adhering, and quickly placed in a tissue basket immersed in cold ice solvent. Generally the isopentane clinging to the samples remained solid during the passage, indicating that the temperature did not rise very much. The samples could usually be shaken free from the foil after a few minutes. They came to rest free inside the basket, and the foil was withdrawn.

Dehydration.—Dehydration was carried out with *n*-butanol [21] at a standard

temperature of − 38°C. The samples were left undisturbed in the cold solvent for various periods and it was found that 48–96 hr. sufficed for most tissues. A central zone of disruption was seen in sections of pieces dehydrated for shorter periods, as described by Freed [7].

Embedding.—After solution of ice, the samples were transferred to test tubes containing fresh *n*-butanol at − 38°C; these were allowed to come to room temperature. The pieces were next transferred to a mixture of *n*-butanol and wax at 48°C. Embedding was carried out both in paraffin wax (m.p. 56°C) and in ester wax (B.D.H.; Stedman [20]). This melted at 48°C.

Mounting of sections.—Five different procedures were used for mounting the microtome sections on slides. Serial sections were taken, and treated as follows: (1) As in normal histological practice they were "floated-out" over water; (2) "floated-out" over physiological saline; (3) over "Intradex" (B.D.H.), an isosmotic solution of highly polymerized glucose in water; (4) over 85 per cent ethanol; (5) "dry-mounted" as in freeze-drying technique.

After floating-out, the sections were gently pressed down on the slides and allowed to dry off slowly at room temperature. The wax was dissolved off in xylol; the sections were passed slowly through absolute and 96 per cent ethanol to denature them, and brought to water through 75 and 50 per cent ethanol for staining and histochemical treatment.

Staining and histochemical procedures.—By deliberate choice, only a relatively simple and standardized stain technique was employed to bring structural details to light. This was a s follows: (1) Harris haematoxylin 1 min. Tap water 2 min. (2) Chromotrope 2R–Fast green–Phosphotungstic acid (Gomori [8]) 1½ min. Rinse tap water. (3) Quick dehydration in 96 per cent and absolute ethanol; xylol; canada balsam.

Alkaline phosphatase was studied by the standard Gomori–cobalt sulphide technique, and polysaccharides by the periodic acid–Schiff method.

RESULTS

Tissues embedded in paraffin wax

The best histological results were obtained after cutting the blocks at, or below, 0°C. This was achieved by cooling microtome, knife and block within a simple perspex cryostat box containing solid CO_2. Conversely, poor results were usual if sections were cut in a warm room. No doubt the wax became too soft; whilst there was no gross effect to be observed during the actual sectioning, the microscopical picture was often very unsatisfactory. Cytoplasm became granular and fine details such as mitochondria disappeared. With pancreas, for example, there was gross distortion and the picture resembled the illustrations given by Lacy and Blundell [14].

Flotation over 85 per cent ethanol, and dry-mounting.—Excellent micro-anatomical and cytological results were obtained with a wide variety of

organs such as pancreas (Fig. 1), liver (Fig. 6), submaxillary gland, kidney (Figs. 4 and 8), and to a less extent intestinal mucosa. The preservation of the cells, their nuclei and mitochondria was extremely good, generally uniformly so over the whole area of the sections. Alkaline phosphatase seemed to be present in its normal distribution in kidney and intestinal epithelial cells and a positive result was obtained with short incubation times (1 min.). Liver glycogen appeared to be normal in quantity and distribution after staining with the periodic acid–Schiff technique.

Flotation over water.—Compared with sections floated over alcohol, those floated over water showed dramatic changes and a striking deterioration in the microscopical appearances. There was an obvious change in the overall staining reaction, which became feebler and less basophilic. Certain cell inclusions, such as submaxillary and pancreatic zymogen granules (Fig. 2), the contents of goblet cells and of ducts, and the remains of plasma within vessels, had disappeared. Mitochondria were affected to a varying extent. In the kidney, for instance, those of the proximal convoluted tubules lost their sharpness of outline and could no longer be resolved properly whilst those of the distal convoluted tubules were hardly affected (Fig. 5). In some organs, especially the pancreas, there was an extraordinary change in the appearance of the cytoplasm; after water flotation, it resembled an empty or vacuolated reticulum (Fig. 2).

Nuclei showed profound changes, which may be best described under two headings. First, their staining reaction had altered. Second, there were quite extreme morphological changes in the majority. Not only had the

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Note.—Unless otherwise stated, photographs were taken with an Ilford 404 filter, sections were of rat tissues, and were stained with the haematoxylin–chromotrope–fast green method described in the text.

Fig. 1.—Magnification, ×500. Pancreas, paraffin wax, alcohol flotation. Zymogen granules, Golgi zone, nuclei, etc. are well preserved.

Fig. 2.—Adjacent section to preceding, floated over water. Zymogen granules have disappeared entirely and cytoplasm has become reticulated. Nuclei show profound changes. ×500.

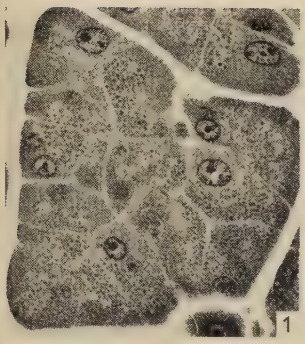
Fig. 3.—Adjacent section to preceding, floated over physiological saline. As before, zymogen granules have disappeared and the cytoplasm appears reticular. Nuclei, though changed in comparison with Fig. 1, do not show the same vacuolation as in Fig. 2. ×500.

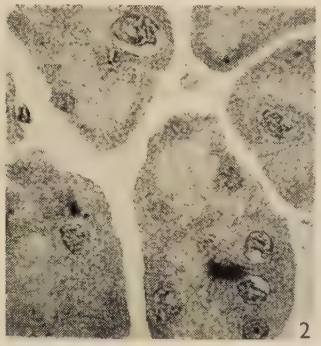
Fig. 4.—Magnification, ×1000. Kidney, paraffin wax, alcohol flotation. To left, a proximal convoluted tubule; to its right, a distal tubule.

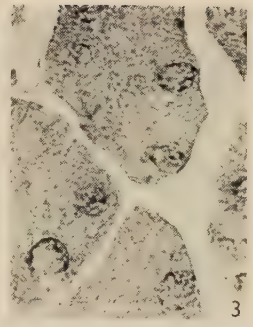
Fig. 5.—Adjacent section to preceding, water flotation. Note disorganization of mitochondria and the nuclear changes. ×1000.

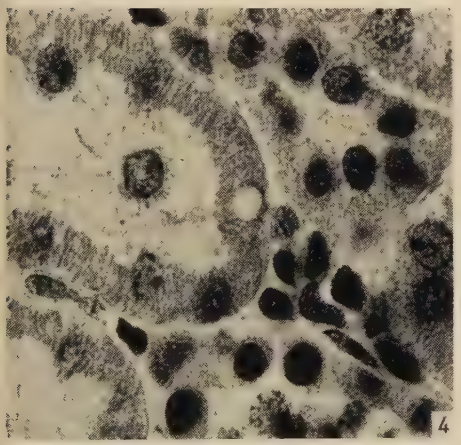
Fig. 6.—Magnification, ×1000. Liver, paraffin wax, alcohol flotation. Stained by periodic acid-Schiff technique. Nuclei, unstained, appear as round empty spaces; glycogen as minute darkly-staining granules in the cytoplasm of the cells.

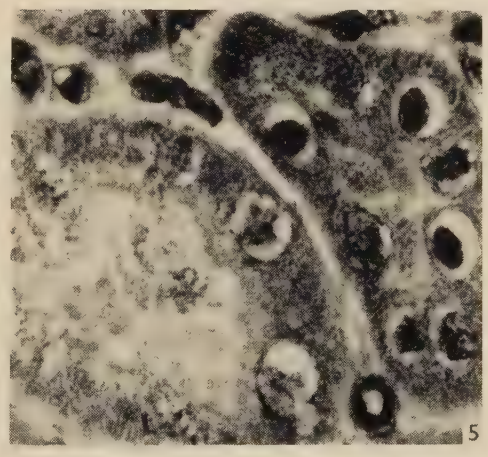
Fig. 7.—Adjacent section to preceding, floated on water, similarly stained. The size and distribution of the particles of glycogen show profound changes. ×1000.

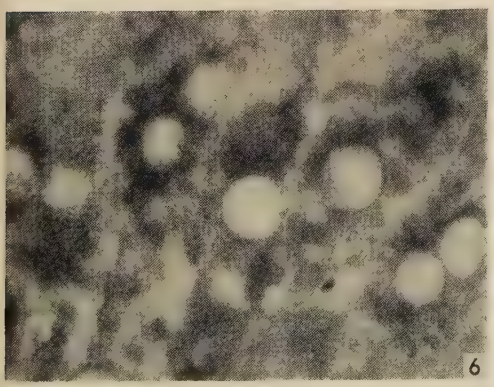


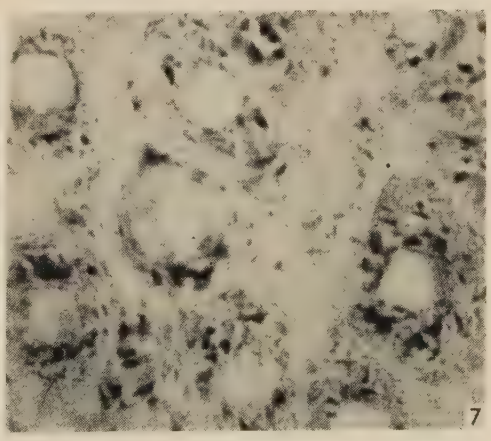












apparent distribution of the chromatin become quite different, but in some there were large spaces, vesicular in appearance, in and around the nuclear membranes. In others, the chromatin had become condensed into a small darkly staining mass (Figs. 2, 5, 9).

After flotating out on water, there were obvious changes in the size, shape and distribution of liver glycogen particles (Fig. 7).

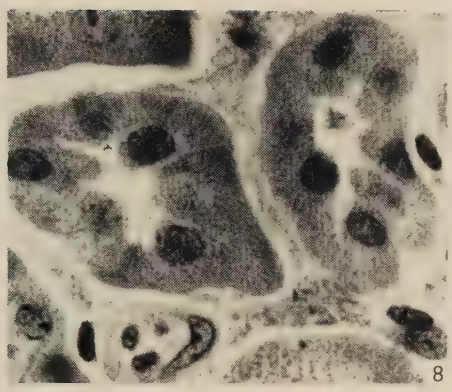
Flotation over saline and "Intradex".—The appearance contrasted both with the alcohol- and water-floated sections. As with water, there was again a loss of overall basophilia, of inclusions such as zymogen granules, and of the contents of ducts. Mitochondria, however, were much less affected. Nuclei, though showing alterations in their staining reaction, did not exhibit the alterations in shape and chromatin distribution seen after water-mounting (Figs. 3, 10).

Tissues embedded in ester wax

Ester wax is, of course, harder than paraffin and refrigeration during section cutting was found to be unnecessary.

There was slight shrinkage in some tissue. Renal tubules and serous-secreting alveoli in the submaxillary gland, for instance, separated slightly. However, the cytological results were even better than with paraffin wax. The fine structure of the cells of, for instance, liver (Fig. 13), kidney (Figs. 11, 12) submaxillary gland (Figs. 17, 21), pancreas (Figs. 14, 15) and intestinal mucosa (Figs. 18, 19, 20) was excellently preserved. Mitochondria were especially clear even with the simple stain employed, whilst a negative image of the Golgi zone was often plainly visible (Figs. 15, 19). With ester wax, it was also possible to prepare sections of embryonic bone cut undecalcified after freeze-substitution; osteoblasts and osteoclasts were well

Fig. 8.—Magnification, × 900. Kidney, paraffin wax, alcohol flotation. Two distal convoluted tubules are shown above an arteriole.
Fig. 9.—Adjacent section to preceding, water flotation. Note the loss of background basophilia; as a result, the cytoplasm of the cells and intertubular constituents appears paler stained. Note also the changes in the nuclei of the tubules. Nucleoli have disappeared; the chromatin pattern has altered; there are vacuolar spaces near nuclear membranes. × 900.
Fig. 10.—Adjacent sections to preceding, floated over Intradex. As in Fig. 9, the cytoplasm is pale and reticular in appearance. Nuclei, whilst altered compared with Fig. 8, do not show the advanced changes seen in Fig. 9. An apparent network of chromatin has appeared; the staining reaction is different. × 900.
Fig. 11.—Magnification, × 900. Ester wax, kidney, alcohol flotation. A transected proximal convolute at bottom of field; two obliquely-cut distals at top. Mitochondria are distinct.
Fig. 12.—Magnification, × 900. Ester wax, kidney, alcohol flotation. Part of a proximal convoluted tubule at top of field; collecting tubule below. Note mitochondria.
Fig. 13.—Magnification, × 900. Ester wax, liver, alcohol flotation. The cells have abundant mitochondria, rod-shaped and granular. Nuclei at bottom left-hand corner belong to a small bile duct.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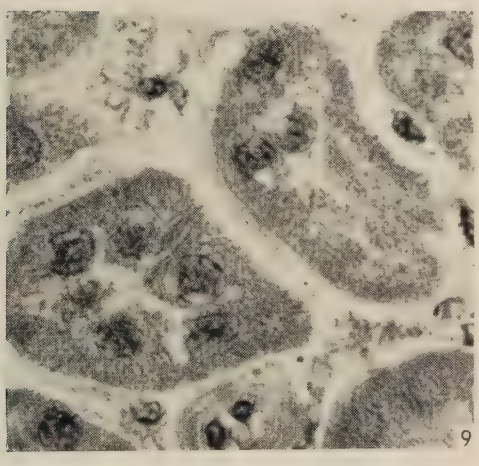
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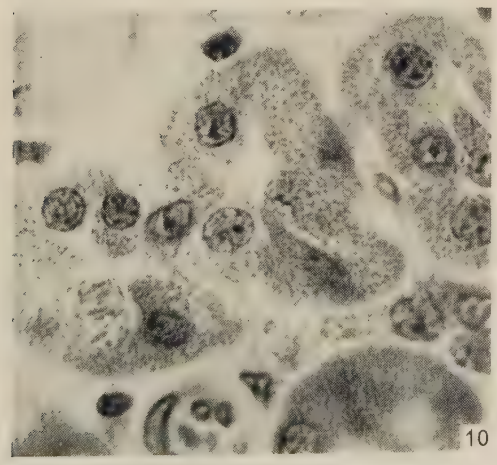
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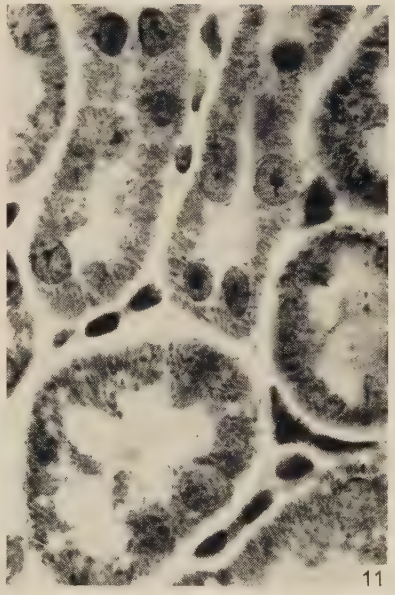
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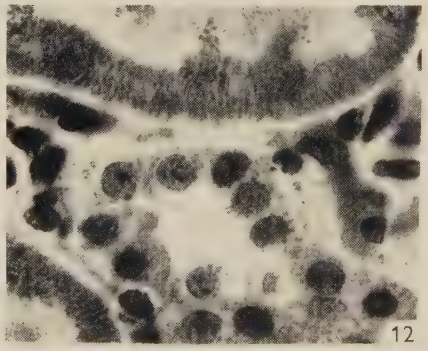
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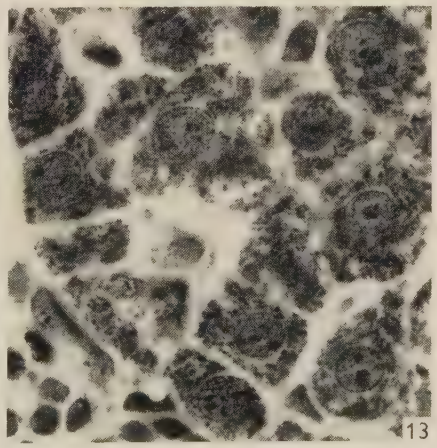
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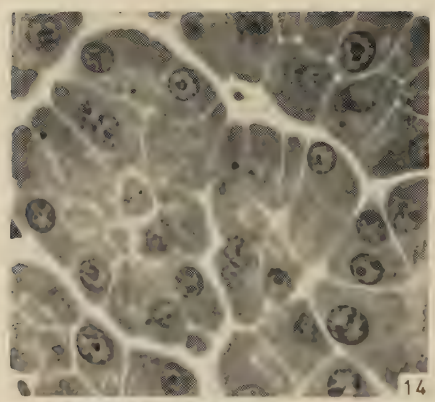
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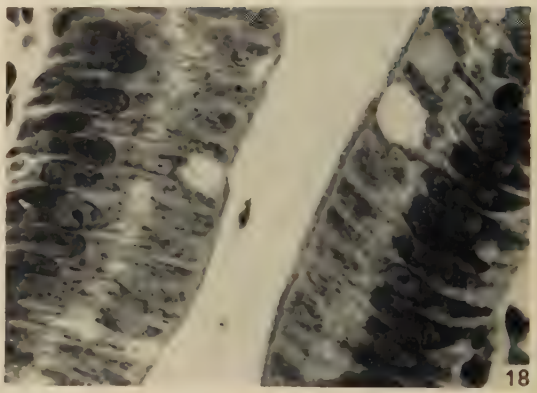
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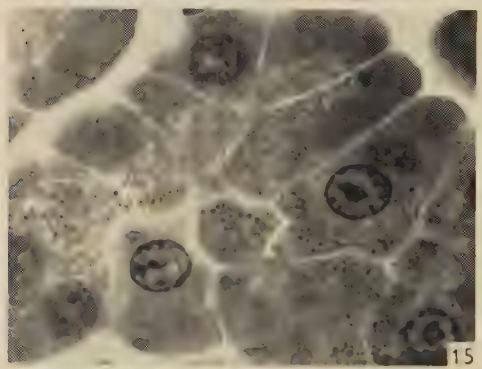
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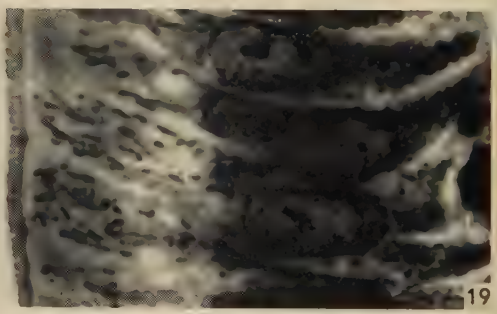
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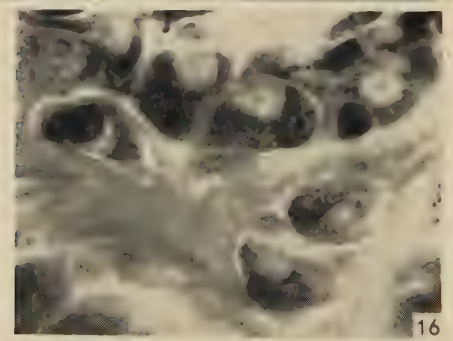
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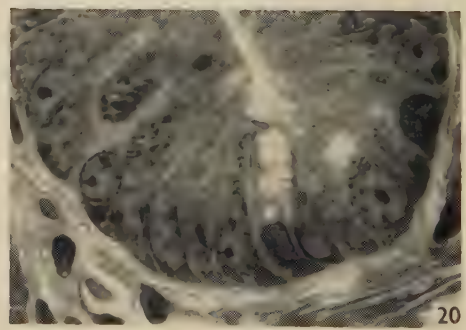
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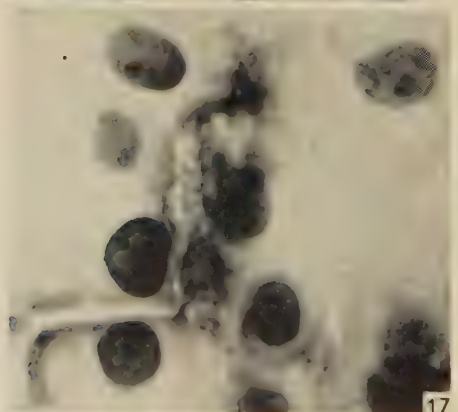
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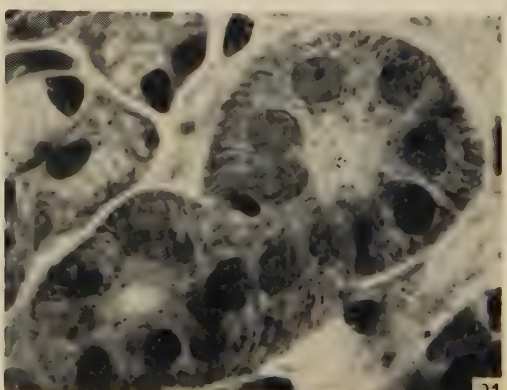
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preserved. Mitochondria and the "juxta-nuclear vacuole" (Golgi area) were easily visible in the osteoblasts (Fig. 16).

Alkaline phosphatase was demonstrable apparently in normal quantity and distribution in kidney and gut; basket cells in the submaxillary gland gave a positive Gomori reaction for the enzyme (Fig. 17). Liver glycogen was present normally.

Ester wax sections were much less affected by floating-out over water. For instance, zymogen granules dissolved very much more slowly, and the nuclear changes, though present, were of lesser degree. The loss of overall basophilia was not so marked as with tissues embedded in paraffin wax.

DISCUSSION

Dealing first with the morphological picture, the results seemed most successful. Although it was not possible to freeze-dry aliquots of all the tissues studied by freeze-substitution, samples of kidney, fowl embryo mesonephros and intestinal mucosa were available for direct comparisons. From study of these, and from scrutiny of the illustrations given by many authors (for instance Pollister and Ornstein [17]), it appears safe to say, with Freed [7], that the results given by the two methods show no significant histological or cytological differences. The preservation and general appearance of the

Fig. 14.—Magnification, ×900. Ester wax, pancreas, alcohol flotation. The pale Golgi zone and the zymogen granules can be distinguished.
Fig. 15.—Magnification, ×1800. Photographic enlargement of portion of preceding. A negative image of the Golgi apparatus is visible in the secretory cells. On the left, it is canalicular in appearance.
Fig. 16.—Magnification, ×1500. Ester wax. Undecalcified embryonic bone. The "juxtanuclear vacuole" (Golgi area) of the osteoblasts can be seen and their mitochondria can just be distinguished. Note also osteocytes below, surrounded by woven bone matrix.
Fig. 17.—Magnification, ×1800. Ester wax, submaxillary gland, alcohol flotation. Gomori-cobalt method for alkaline phosphatase. Incubation time, 1 hr. The fibre-like processes of a basket cell are present in the centre of the field, spreading out above and below from the region of its nucleus. The latter is oval, rather small, and partly out of focus. Both nucleus and processes are blackened, indicating that they contained alkaline phosphatase. The processes terminate around and upon the secretory cells whose outlines are just visible. The secretory nuclei are also blackened, but this is a diffusion artefact; it will be seen that the poles nearer the basket cell are the darker.
Fig. 18.—Magnification, ×930. Ester wax, small intestine, alcohol flotation. An intervillous space with two rows of epithelial cells. Note filamentous supranuclear and granular infranuclear mitochondria. The Golgi zone appears as a pale supranuclear area.
Fig. 19.—Magnification, ×1800. Photographic enlargement of portion of above. A canalicular structure can be distinguished in the pale supranuclear Golgi zone, especially in centre and bottom of picture. Filamentous mitochondria are present in the supranuclear cytoplasm, granular in the infranuclear.
Fig. 20.—Magnification, ×930. Ester wax, small intestine, alcohol flotation. A Lieberkühn crypt, with Paneth cell. The large epithelial nucleus to its right was evidently crystallised during quenching.
Fig. 21.—Magnification, ×930. Ester wax, submaxillary gland, alcohol flotation. A duct is shown, with secretory cells above and to right. Note mitochondria.

tissue in the microscope was at least equal to that given by the best classical chemical fixatives.

Perhaps the most interesting finding was that, after paraffin wax embedding, appearances were so dramatically influenced by the mounting procedure. Clearly, the sections must have been permeatad by the fluids used for floating them out. It is, of course, well known that water permeates floating paraffin wax sections to some extent; most histologists, for instance, are familiar with the yellow colouration which appears in the water used for floating-out sections of tissue fixed in Bouin's fluid. Kruszynski [13] showed by microincineration technique that inorganic constituents dissolve from paraffin sections into water used for floating them out; water flotation is, therefore, to be avoided in microincineration [11]. Holt *et al.* [12] have reported that Feulgen-positive material and alkaline phosphatase diffuse out from paraffin sections floating on water.

Thus, the excellent pictures obtained after floating-out over alcohol must have been due to the action of the latter on the sectioned tissue, through which it permeated. This evidently brought about a denaturation and chemical fixation of the tissue, abolishing its sensitivity to the watery reagents with which it later came into contact. Alcohol flotation is, of course, used extensively in work with frozen-dried tissue for this reason.

A further point of interest was the differential effect of water flotation on mitochondria of renal, distal and proximal convoluted tubules. The latter were much less affected, a fact which must reflect differences in their physicochemical constitution. Mitochondrial changes were less pronounced after isosmotic flotants had been used; this correlates with the preservation of mitochondria after differential centrifugation in saline solutions [2].

The effects on the sections of water flotation seem to fall into two categories. First, there was the disappearance of structures such as zymogen granules, the contents of goblet cells and of ducts, and the decline in overall basophilia. The latter was observed also by Freed [7]. This indicates most strongly a loss of soluble materials into the flotant. Second, there were the changes in mitochondria and the curious alterations in the nuclei of sections floated on water. These showed not only changes in staining reaction, evidence of loss by solution, but also changes in morphological appearance which could scarcely have been the result of solution loss. Since such changes did not occur when isosmotic flotants were employed, it seems more than probable that the tissue was sensitive to the osmotic environment.

Loss by solution from ducts and goblet cells, and from the section generally as shown by the changed staining reaction, indicate that soluble materials were preserved by freeze-substitution; this is what would be expected.

However, loss of zymogen granules, presumably by solution, has deeper implications. Zymogen granules are, of course, preserved in tissue treated with orthodox watery fixatives (formol, etc.); but they do not dissolve when sections are floated over water. This is presumably because their chemical nature is altered by the fixative. The fact that the granules dissolve after freeze-substitution suggests very strongly that they survive the procedure more or less chemically unchanged. This point will be investigated by cytochemical methods. These facts, together with the apparent sensitivity of freeze-substituted tissue to the osmotic environment, suggest that such material escapes without denaturation. It therefore follows that freeze-substitution does not entail a frank chemical fixation such as occurs with tissue immersed in alcohols at room temperature. The reasons for this must next be discussed briefly.

The gross shrinkage and distortion following exposure to alcohol at room temperature probably arise from two main causes. On the one hand, from a violent and abrupt removal of water, no doubt accompanied by quite powerful and disruptive streaming movements in the tissue. On the other, from a sudden denaturation and frank coagulation of protein whose molecules contract. In freeze-substitution, conditions are entirely different. At the low temperatures involved, in the first place, the water is held as ice and the tissue viscosity has fallen to zero. The ice simply dissolves slowly in the solvent; there are no disruptive streaming movements. In the second place, the denaturing effect of the alcohol is, apparently, practically abolished. This is presumably a function of temperature; it is known that at temperatures around $-5°C$ plasma proteins are not denatured by quite high concentrations of ethanol [5]. Nevertheless, it should be borne in mind that some degree of irreversible denaturation must almost certainly occur during the "warm-up" stage when the tissue, after solution of its ice in cold solvent, is allowed to come to room temperature. This is probably the explanation for the fact that whilst water-floated sections show extensive changes they do not disintegrate and dissolve completely as do sections of frozen-dried material. Clearly, the warm-up stage should be kept as short as possible.

It is interesting that ester wax sections were relatively resistant to water flotation. This unexpected finding may indicate that water does not permeate ester wax sections or that water soluble substances and components liable to osmotic changes are altered by contact with the molten wax. Against the former is Stedman's [20] report that ester wax sections are actually permeable to water. Against the latter is the lower melting point of ester wax (48°C) compared to that of the paraffin wax employed (56°C). There remains the further possibility that the "wetting power" of ester is greater than that of

paraffin wax. If this were so, sectioned tissue might be more firmly bonded and protected by the former. However, there is insufficient evidence to justify any definite explanation at present.

We come now finally to the indications for freeze-substitution in cytology and cytochemistry. In practice, this question resolves itself into a discussion of its merits relative to the only other comparable technique, namely, freeze-drying. For the sake of brevity, it is proposed to discuss microscopic investigations under three headings. First, there is work in which, after dissolving the wax, sections are brought to water or to lower grades of alcohol for further treatment. This would cover, for instance, the use of classical staining methods; of histochemical tests with watery reagents; and the study of optical path differences relative to water. For many purposes of this sort, freeze-substitution would recommend itself on grounds of simplicity and reliability. Freeze-drying would offer no advantages, since the sections would, in any case, have to be fully denatured to withstand solution by the watery reagents. Moreover, there is the added expense and trouble involved with the high vacuum equipment. There might, however, be two circumstances in which freeze-drying would be preferred. First, some particularly sensitive substance, perhaps an enzyme, might conceivably be damaged by sojourn in alcohol even at the low temperatures involved, or during the warm-up period. Second, there is the question of the extend to which substances dissolved in the tissue and cellular fluids, before quenching, might be artificially redistributed in space during solution of ice. This would apply more particularly, perhaps, to the smaller molecules. However, similar doubts must apply where ice is sublimed off, as in freeze-drying.

Second, there is work in which the wax is dissolved and the sections are directly mounted without further treatment. Investigations of this sort generally depend upon phase-contrast [10] or interference microscopy [9]. For simple morphological studies there would not seem to be any advantages in freeze-drying. However, for quantitative work with the interference microscope, freeze-drying might be the safer method; the partial denaturation accompanying the warm-up stage of freeze-substitution might conceivably alter optical path differences and thus introduce a variable factor. This point is being investigated further.

Third, there is the study of the ash left by microincinerating sections from which wax has been dissolved. Obviously, the basis for critical investigation upon spodograms is that the mineral constituents must be unaltered in distribution and concentration. Work is in progress to determine whether these criteria are fulfilled as well by freeze-substitution as by freeze-drying.

SUMMARY

1. Rat tissues were "freeze-substituted" by means of a thermostatically-controlled apparatus employing mechanical refrigeration. The latter is described.

2. The tissues were embedded in both paraffin and ester waxes. Serial sections were "floated-out" over 85 per cent ethanol, water, physiological saline and Intradex. Others were dry-mounted. The cytological and histochemical results were compared.

3. With ethanol, excellent results were obtained, especially after ester wax embedding. The preservation equalled that obtained with freeze-drying.

4. With water, there was a profound deterioration. Overall basophilia decreased sharply and the contents of ducts, and zymogen granules, disappeared, presumably by solution. In addition there were extreme morphological changes in nuclei and mitochondria.

5. With saline or Intradex, there were again solution losses. However, the morphological changes in nuclei seen after water flotation were absent. Hence it is inferred that they were osmotic in nature.

6. On these and other grounds, it is concluded that protein denaturation is practically absent after freeze-substitution.

7. The respective merits of freeze-substitution and freeze-drying, and their applications in cytology and cytochemistry, are discussed.

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SOCIAL BEHAVIOUR OF CELLS IN TISSUE CULTURE

III. MUTUAL INFLUENCE OF SARCOMA CELLS AND FIBROBLASTS

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Received March 3, 1957

It has been shown in an earlier paper [4] that fibroblasts in tissue culture interfere with each other's movement when they make mutual contact. Movement of a cell in the direction of a contact tends to be prevented, a phenomenon termed "contact inhibition". One of its effects is to hold a fibroblast at a standstill when it is so closely surrounded by other fibroblasts that the only surfaces available for its movement are those of its neighbours. Weiss [18], surveying the perspectives of morphogenesis, had earlier suggested that such an effect might occur. It seems probable that this is one of the mechanisms, though not the only one [1, 2], that hold fibroblasts stationary in the normal adult. Not all types of cells remain immobile when surrounded by other stationary cells; in particular those cells which have become malignant invade their surroundings. If mutual inhibition of movement by contact plays a significant part in immobilising fibroblasts then it might be expected to be absent between sarcoma cells and the normal fibroblasts amongst which they have arisen. Preliminary qualitative tests gave some support to this conjecture, since no contact inhibition between cells of a mouse sarcoma and various fibroblasts could be detected [5]. Such qualitative observations are however imprecise and somewhat subjective. The present paper provides necessary quantitative documentation.

It is usually impossible to test a sarcoma against its tissue of origin; and moreover when demonstrating a lack of contact inhibition between sarcoma cells and normal fibroblasts one must reckon with the possibility that the cells of origin might likewise be uninhibited by contact with the fibroblasts used for testing. All that we can hope to do is to show that a wide range of fibroblasts inhibit each other and do not inhibit sarcoma cells. Qualitatively we have observed this to be so with a considerable number of different fibroblasts; but in the present paper we have concentrated on a detailed analysis of two widely different types of fibroblasts, those obtained from embryo chick heart and those from neonatal mouse skeletal muscle. These two types manifest contact inhibition against each other but fail to inhibit the movement of two types of mouse sarcoma.

METHOD

Interaction of cells was observed by the method of confronted cultures: two explants were placed near enough for their outgrowths to meet. The explants were primary ones of the following kinds: (1) from the hearts of chick embryos incubated 6–13 days, (2) from skeletal muscle of 1–2 day old mice, (3) from tumours of mouse sarcoma 37, or mouse Crocker sarcoma (sarcoma 180). The two sarcomata were subcutaneously transplanted strains obtained in the first instance from the Chester Beatty Research Institute. Coverslip cultures were prepared in which the cells moved on a plane surface in a liquid medium of chick embryo extract diluted with Pannett and Compton saline. The plane surface was not that provided by the coverslip itself, but by a thin clot of fowl plasma and embryo extract on which the explants were laid. It was found that the sarcoma cells migrated much better on this surface than directly on glass. Explants were placed ½ to 1 mm apart, usually several in a linear row. Neighbouring explants were from different sources. The two different sarcomata were not tested against each other. Since the mouse muscle explants had a latent period about 24 hours longer than that of either the chick heart or the sarcoma explants, the mouse muscle was placed on the clot surface first, the culture was incubated overnight and the other explants of sarcoma or chick heart were then added. The progress of the living cultures was watched from time to time, but the quantitative observations were made on cultures fixed in formalin, 24–72 hours after setting up or adding the second explants, and stained with alum haematoxylin.

RESULTS

Qualitative description

The cells from the different explants are usually readily distinguishable. A chick heart fibroblast is usually more flattened on the surface of the clot than a mouse muscle fibroblast, which commonly has many long cytoplasmic processes and is more deeply stained (Fig. 1). The sarcoma cells are of two types (Fig. 2), though intermediate forms occur, and we have observed transformation of one to the other (see also [12] and [14]). The first type is the rounded form, very refringent in the living state and particularly deeply stained in the fixed preparations. It can readily be distinguished from the smaller, more irregular, macrophages [15] which are sometimes common. The second type is the spindle form with polarised cytoplasm. These cells are less flattened than are the normal fibroblasts and their whole form is more compact. Extreme spindle types may be fibroblast-like but can usually be distinguished from fibroblasts in the same culture by their greater depth of staining. We believe that there are quantitative differences in the behaviour of the rounded and spindle forms, but for the purposes of the present analysis they have not been treated separately. They occur in varying proportions in cultures of both Crocker and S 37.

The evolution of cultures consisting of a sarcoma confronted with a chick heart or mouse muscle explant is quite different from that of cultures consisting of a chick heart confronted with a mouse muscle explant. In the former, from the start, the sarcoma cells migrate more extensively towards the fibroblast explant than they do from the sides of the sarcoma explant away from the fibroblast explant. There is no obvious hesitation in the movement of either fibroblasts or sarcoma cells when they meet between the two explants; the sarcoma cells move over the surface of the fibroblasts that is exposed to the fluid medium, and after two days of culture some of them have almost always reached the fibroblast explant. Both round and spindle forms take part in the invasion, but the round usually predominate and precede the spindle forms. Crocker and S 37 explants behave identically. The fibroblasts move under the outgrowth of sarcoma cells, and in some cases reach the sarcoma explant. When mouse muscle is confronted with chick heart both outgrowths, before they meet, often extend more towards each other than to the sides. When the two sheets of fibroblasts meet each other, however, there is no obvious migration of one over the other. The progress of both outgrowths on the contrary is obstructed by their junction. The mouse muscle cells become much more closely packed together between the two explants than at the sides; but the chick heart fibroblasts, normally closely packed, do not appear to become much more so when they have met the muscle cells. There is a certain amount of intermingling or interdigitation of the two outgrowth in the zone of junction but both kinds of fibroblast tend to remain in a single layer.

Quantitative observations

Measurements were made on sample strips of the zone of outwandering from each explant. The strips were 100 μ wide, and extended radially from the edge of the explant as far into the medium as cells from that explant had travelled. A strip was placed in the radius directed towards the confronting explant ("between") and in the radii at right angles to this, directed towards unoccupied medium ("side").

The following measurements were made on each strip: (1) The *strip population*, i.e. the total number of cells in the strip. (2) The *outgrowth length*, i.e. the radial length of the strip. (3) The *population density*, i.e. the strip population divided by the area of the strip. When the outgrowth was sufficiently extensive (4) the *central density*, in the 170 μ of radial length of the strip next to the explant and (5) the *peripheral density*, in the 170 μ length at the peripheral end of the strip, were also determined. Central densities of all

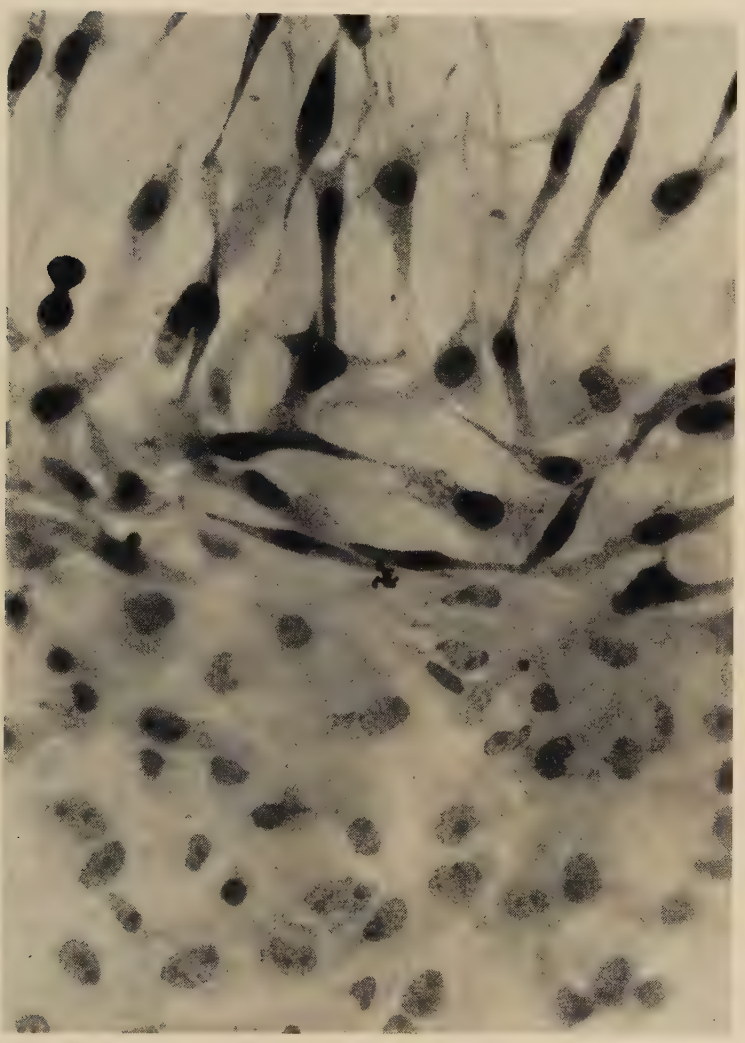

Fig. 1.—Zone of junction of mouse muscle fibroblasts (above) and chick heart fibroblasts (below). × 475.

cell types at the sides of the explants are about five times higher than peripheral densities.

The influence on outward migration produced by a confronting explant has been expressed by comparing measurements made on the "between" strip with those made on the "side" strips. Ratios of the former to the latter, to be referred to as between/side ratios, form the main material for the analy-

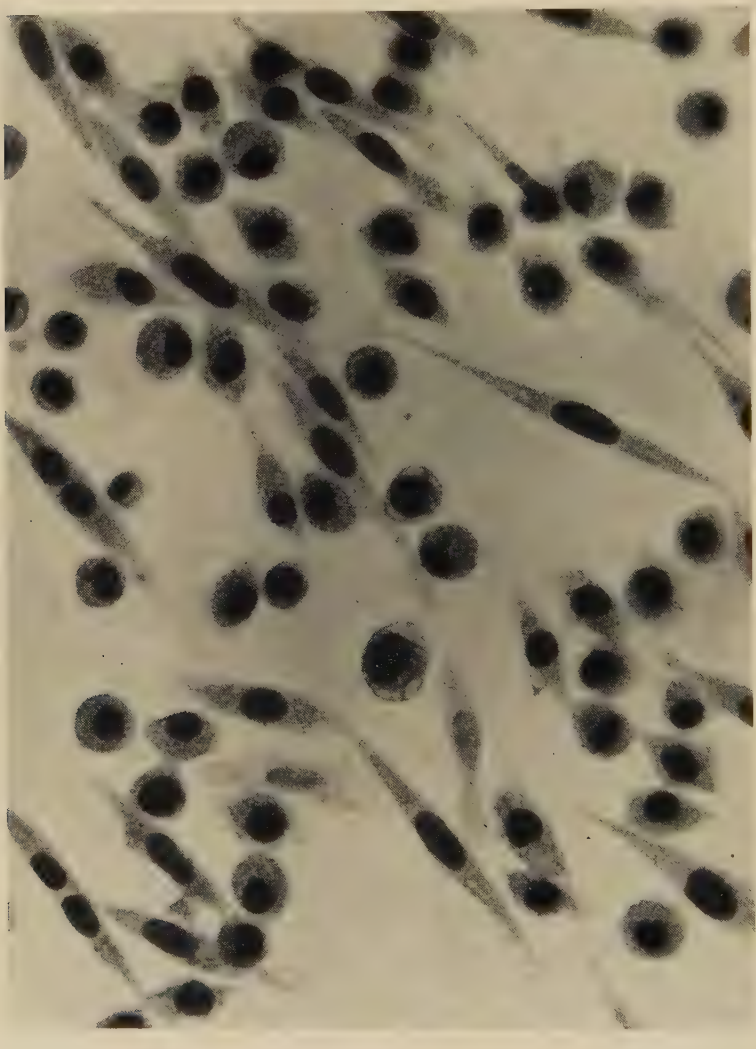

Fig. 2.—Rounded and spindle forms of sarcoma cells (S37) with intermediate forms. ×475.

sis. Any departure of such a ratio from unity represents a distortion of the outgrowth. The ratios of outgrowth lengths indicate distortions of the pattern of cell movement. The ratios of strip populations and of population densities may in addition indicate effects on the incidence of mitosis, but these we have not yet attempted to distinguish.

In order to separate any effects exercised by the opposed explant via the

TABLE I

The upper half of the table compares the outgrowth of the two kinds of sarcoma when confronted with mouse muscle, after junction of the outgrowths. The lower half shows the corresponding outgrowth of the mouse muscle when confronted with the two kinds of sarcoma. Means and their standard errors are given. Ratios are between/side, and those marked * are significantly different from unity at the 5 per cent level. The row headed "Difference, *P*" is in each case the result of comparing the two overlying means, expressed as a probability that they are drawn from the same population. *N* is number of cultures. Outgrowth length is expressed in units of 10 μ, population density as cells per 1000 μ^2.

	N	Strip population			Outgrowth length			Population density		
		Between	Sides	Ratio	Between	Sides	Ratio	Between	Sides	Ratio
					Sarcoma versus muscle					
	12	98±17	45±7	2.05±.26*	45±6	26±6	2.13±.47	2.37±.28	2.19±.28	1.19±.16
ker	13	102±19	68±13	1.77±.28*	68±9	37±6	1.94±.14*	1.51±.18	1.78±.15	.88±.09
erence, *P*		>.2	>.2	>.2	.5	>.2	>.2	.02−.01	.2 −.1	.2 −.1
					Muscle versus sarcoma					
us S 37	12	80± 8	67± 7	1.25±.09*	80±6	109±7	.82±.13	1.22±.20	.69±.09	1.89±.31*
us Crocker	13	85±11	74±11	1.19±.07*	86±4	110±9	.85±.08	1.08±.15	.74±.10	1.50±.12*
erence, *P*		>.2	>.2	>.2	>.2	>.2	>.2	>.2	>.2	>.2

medium (either clot or liquid phase) from those produced by actual contact with the opposing cells, we have made a separate analysis of cultures fixed before the two outgrowths had met, and after their junction. At the sides, where they are uninfluenced by the opposing explant, the mean outgrowth lengths and strip populations in the after junction group are 1½–2 times higher than in the before junction group; the overall population densities hardly differ.

Sarcoma.—The first point we sought to establish was that the two varieties of sarcoma need not be treated separately in the analysis. The most extensive test of this supposition was made on cultures of sarcoma versus mouse muscle after junction of the outgrowths (Table I). The between/side ratios are in no case significantly different. In absolute measurement S 37 has however a significantly greater density and a shorter linear outgrowth between explants than has Crocker sarcoma. The lower half of Table I demonstrates that the two kinds of sarcoma do not induce a significantly different behaviour in the mouse muscle cells confronting them.

Since we shall be mainly concerned not with the absolute measurements but with the between/side ratios of these measurements, it is legitimate to simplify our analysis by pooling the results from the two sarcomata. In

TABLE II

Outgrowth of sarcoma cells opposed by fibroblasts.

Mean between/side ratios and their standard errors are given. For other conventions see legend to Table I.

	N	Strip population	Outgrowth length	Population density
Versus mouse muscle				
Before junction	13	$1.56 \pm .28$	$1.56 \pm .24$*	$1.39 \pm .37$
After junction	25	$1.90 \pm .20$*	$2.03 \pm .24$*	$1.03 \pm .09$
Difference, *P*		$> .2$	$> .2$	$> .2$
Versus chick heart				
Before junction	11	$2.4 \pm .8$	$1.62 \pm .28$*	$1.36 \pm .33$
After junction	8	4.4 ± 1.3*	$2.13 \pm .27$*	$2.12 \pm .57$
Difference, *P*		$.1 - .2$	$> .2$	$> .2$

Table II the behaviour of the sarcoma cells, regardless of type, is analysed when confronted with one or other of the two types of fibroblast. Against mouse muscle there is already before junction a tendency, which is significant, to grow linearly further towards the opposing explant than towards the sides. After junction this tendency is if anything more pronounced, though not significantly more so. The population density is not significantly affected by this distortion of the outgrowth; the strip population is significantly affected after junction but not before.

Against chick heart fibroblasts the sarcoma cells behave in much the same way as they do against mouse muscle cells. Indeed before junction there is no significant difference in the effect of the two kinds of fibroblast. After junction there is still no difference of effect on outgrowth length. But the between/side ratio for sarcoma cell density is now significantly higher against chick heart than against mouse muscle (t 3.0, d.f. 32, $P < 0.01$); and the same consequently holds for the strip population (t 3.1, d.f. 32, $P < 0.01$). Since there are no significant differences before junction, the groups of sarcoma cultures confronted with chick heart and with mouse muscle can be combined into a general group of sarcoma versus fibroblasts. The strip population ratio of this combined group is significantly greater than unity (1.9 ± 0.4), so it may be concluded that an asymmetry of cell numbers as well as of distance migrated is produced before junction by an opposing fibroblast explant. The population density cannot be shown by the same means to be significantly distorted before junction: but if the density ratios for sarcoma versus chick heart both before and after junction are combined, the resulting

TABLE III

Outgrowth of mouse muscle fibroblasts opposed by sarcoma cells or chick heart fibroblasts. Mean between/side ratios and their standard errors are given. For other conventions see legend to Table I.

	N	Strip population	Outgrowth length	Population density Total	Peripheral	Central
Versus sarcoma						
Before junction	13	1.15 ± .14	1.13 ± .09	1.08 ± .13	1.5 ± .6	1.38 ± .18
After junction	25	1.22 ± .06*	.84 ± .07*	1.69 ± .16*	2.8 ± .4*	1.45 ± .12*
Difference, *P*		> .2	.02 – .01	.02 – .01	.1 – .05	> .2
Versus chick heart						
Before junction	17	1.47 ± .13*	1.36 ± .16*	1.22 ± .12	1.5 ± .3	1.38 ± .14*
After junction	19	1.31 ± .12*	.76 ± .07*	2.00 ± .25*	5.7 ± 1.1*	1.21 ± .14
Difference, *P*		> .2	< .01	.02 – .01	< .01	> .2

ratio proves to be significantly greater than unity (1.7 ± 0.3). At some period in the life of a culture, therefore, the density of sarcoma cells between the explants is relatively increased by confronting fibroblasts.

Mouse muscle fibroblasts.—In Table III the results of the analysis of the behaviour of mouse muscle fibroblasts opposed either by sarcoma cells or by chick heart fibroblasts are set out. Before junction an opposing sarcoma explant does not significantly distort the fibroblast outgrowth. After junction however, the between/side ratio of outgrowth length has become significantly less than unity, because the outward migration of the fibroblasts has been hindered between the explants. The overall density of population after junction is considerably higher between the explants than at the sides, the ratio being significantly greater than before junction. But this is not merely a result of the shorter length of outwandering between the explants. After junction the strip population between the explants is significantly greater than that at the sides.

When opposed to chick heart, in contrast to the behaviour against sarcoma, there is an obvious distortion of the mouse muscle fibroblast outgrowth before junction (Table III). This takes the form of a greater length of outgrowth and a greater strip population between the explants than at the sides. The density ratio is also greater than unity, but not significantly so except in the central zone. After junction the between/side ratio of overall densities becomes significantly greater than unity, and significantly higher than before junction. The strip population ratio does not change. But after junction,

TABLE IV

Outgrowth of chick heart fibroblasts opposed by sarcoma cells or mouse muscle fibroblasts. Mean between/side ratios and their standard errors are given. For other conventions see legend to Table I.

	N	Strip population	Outgrowth length	Population density		
				Total	Peripheral	Central
Versus sarcoma						
Before junction	11	1.19 ± .12	1.18 ± .13	1.08 ± .09	1.08 ± .14	1.11 ± .12
After junction	9	1.10 ± .12	.89 ± .09	1.24 ± .09*	1.57 ± .33	1.15 ± .09
Difference, *P*		> .2	.2 – .1	> .2	> .2	> .2
Versus mouse muscle						
Before junction	16	1.31 ± .16	1.14 ± .11	1.13 ± .06	1.14 ± .14	1.21 ± .13
After junction	19	.83 ± .06*	.68 ± .05*	1.23 ± .06*	1.45 ± .18*	1.05 ± .06
Difference, *P*		< .01	< .01	> .2	> .2	> .2

as when confronted by sarcoma, the between/side ratio for length of outgrowth falls significantly below unity. A comparison of the outgrowths after junction against the two types of opposing cell shows that the only ratios that differ significantly (P 0.02 – 0.01) are those of peripheral density: confrontation by chick heart produces a remarkably high peripheral density ratio.

Chick heart fibroblast.—When in opposition to sarcoma (Table IV) the between/side ratios have much the same values as have mouse muscle fibroblasts confronted with sarcoma. But, perhaps because the sample is a smaller one, the only between/side ratio significantly different from unity is that for overall population density after junction: density is higher between the explants. The small sample size probably also accounts for the lack of difference between the ratios before and after junction.

Chick heart confronted with mouse muscle exhibits before junction no significant distortion of outgrowth in any of the characteristics measured. After junction it has suffered the same obstruction to the linear extension of its outgrowth as has mouse muscle opposed by chick heart; but there is a different effect on density and strip population. The between/side ratio of density, instead of rising sharply as does that of mouse muscle, does not change significantly after junction. The between/side ratio of population, instead of remaining constant, falls sharply after junction. The obstruction in other words seems to check the whole process of outwandering without forcing the cells more closely together. Chick heart fibroblasts are normally more densely packed than are mouse muscle fibroblasts: in the unobstructed

outgrowth at the sides the density of population is about 3 times higher in the former than in the latter.

Before junction the effect of opposition by mouse muscle does not differ significantly from that by sarcoma cells. It is possible therefore to combine the data and demonstrate that when faced by a second explant the strip population of chick heart is significantly greater between explants than at the side (the between/side ratio is 1.26 ± 0.11) and that the increased density too is on the border line of significance (the between/side ratio is 1.11 ± 0.05). After junction the between/side strip population ratio is significantly less when opposed to mouse muscle than to sarcoma (P 0.05 − 0.02), and the length of outgrowth is also less, though the difference lies only on the borderline of significance (P 0.05).

Comparisons of cell types.—The most critical demonstration of the difference between sarcoma cells and fibroblasts can be got by comparing their behaviour against the same type of opposing explant. When both sarcoma and mouse muscle are opposed to chick heart, none of their between/side ratios differs before junction ($P > 0.2$). After junction there are highly significant differences ($P < 0.01$) in the ratios of strip populations and of lengths of outwandering, the sarcoma having extended much more between the explants than at the sides, the mouse muscle much less. The density ratios do not differ significantly ($P > 0.2$). Precisely the same relations hold when one compares sarcoma cells with chick heart fibroblasts, both being confronted by mouse muscle fibroblasts.

Finally the reciprocal behaviour of different cell types when influencing each other can be compared. We can contrast the pattern of growth of fibroblasts of both types combined together (they do not differ significantly) when opposed to sarcoma cells with the reciprocal pattern of sarcoma cells when opposed to fibroblasts. It emerges that the outgrowth of the sarcoma cells is significantly more distorted before junction by a fibroblast explant than vice versa, in outgrowth length (P 0.05 − 0.02) but not in strip population (P 0.10 − 0.05) nor in density (P 0.3 − 0.2). The combined fibroblast data in fact shows no significant between/side distortion before junction when confronted with sarcoma. After junction the differences in strip population and in outgrowth length ratios are exaggerated by the obstruction which the fibroblasts but not the sarcoma cells meet between the explants, and both are highly significant. The density ratios again do not differ significantly.

A comparison of mouse muscle against chick heart with the converse shows no significant difference in distortion of the outgrowth before junction. It is possible therefore to combine the data and to show that besides strip

TABLE V

Mutual overlapping of cells of different types in the region between two explants.

"Nuclear points" records the number of times that nuclear centres of the first cell-type in the left hand column overlapped nuclei of the second cell-type ("hits"), or failed to do so ("misses"). "Random points" is a similar record, but with randomly distributed points used instead of the nuclear centres of the first-cell type.

	No. of cultures	Nuclear points Hits	Nuclear points Misses	Random points Hits	Random points Misses	$\chi^2_{[1]}$	P
Sarcoma on mouse muscle	12	81	656	97	671	0.980	.3 – .5
Chick heart on mouse muscle	18	25	1,019	68	691	36.34	< .001
Sarcoma on chick heart	10	117	437	121	519	0.919	.3 – .5
Mouse muscle on chick heart	18	16	657	78	681	38.86	< .001

population and outwandering length, total density in fibroblast cultures confronted with other fibroblasts is significantly higher between explants than at the side (combined ratio 1.17 ± 0.07). After junction outgrowth length is similarly depressed between the explants in both fibroblast types, but the strip population and density ratios are highly significantly greater in mouse muscle than in chick heart fibroblasts.

Overlapping of cells.—The measurements so far described show that two opposed fibroblast outgrowths obstruct each other when they have made contact, but that a fibroblast outgrowth does not obstruct a sarcoma outgrowth. In order to decide what part contact inhibition plays in this difference, it is necessary to know how much the two kinds of cell opposed in a culture are superimposed on each other in the region where the outgrowths have joined. The mutual overlapping of any two types of cell in this region was estimated as follows. The proportionate area of the culture surface occupied by nuclei of cell-type A was obtained by the method of Chalkley [7]. The mid-point of each nucleus of cell-type B was then examined, and the proportion situated on or underneath nuclei of cell-type A recorded. The two proportions were then compared. The results are given in Table V. It is evident that the superimposition of sarcoma nuclei on fibroblast nuclei is no different from a random distribution; but the superimposition of either kind of fibroblast nuclei on the other kind is very much less than is to be expected by chance. Several cultures were used for each comparison; there was no significant heterogeneity between cultures.

DISCUSSION

Influences exerted before junction

The main purpose of this paper was to determine the amount of contact inhibition between sarcoma cells and fibroblasts when they are caused to meet by juxtaposing two explants. The results have shown however that there are mutual influences exerted by the two explants at a distance, before any contact has been made between the two outgrowths, and these must be discussed first.

Before junction, there is a larger outgrowth of mouse muscle, both in terms of distance moved and of total cell population, towards a chick heart explant than in a direction not towards it. The converse effect, the exaggerated outgrowth of chick heart towards mouse muscle, was not demonstrated conclusively by our data; there was however a significantly greater outgrowth towards an opposed explant regardless of its nature, whether mouse muscle or sarcoma. It is obviously appropriate to interpret such a distortion of growth as an example of the "two centre effect" as analysed by Weiss [19], the result of contact guidance [17] by orientated fibrin micellae in the plasma clot surface between the two explants. Such an interpretation is reinforced by the fact that no sign of a two centre effect was previously detected [4] between chick heart explants grown on a glass surface, which cannot be orientated. The effect of contact guidance away from the explant should be to increase the net speed of outward movement [20], and hence the outgrowth length. If the interpretation of our results as an effect of contact guidance is right, then an increased density of population is also a result, perhaps because the individual cells are more compact in form when orientated. The number of cells in a radial strip is increased too, presumably by a greater number of cells leaving the explant in unit time. This is explicable by a relaxation of the incidence of contact inhibition on the emerging cells both by the more rapid centrifugal movement and the greater compactness of the cells in the outgrowth. A change in mitotic rate as a result of contact guidance has not, however, been excluded.

Prima facie it seems likely that the strong "attraction" of sarcoma cells towards a fibroblast explant is also an instance of the two centre effect, produced by contact guidance. In such cultures there are signs that the fibroblasts are also being conversely "attracted" to the sarcoma explant, which supports the view that orientation is present in the medium. Such an effect on the fibroblasts before junction was not statistically significant,

but the significantly increased between/side ratio for strip population found *after* junction for mouse muscle versus sarcoma is very difficult to explain except as the late result of a two centre effect. There are however indications that judgement should be suspended about the nature of the disproportionate outgrowth of sarcoma cells towards a fibroblast explant. In the first place the sarcoma outgrowth is very much more distorted than is the fibroblast outgrowth opposite it; if contact guidance is responsible, the sarcoma cells must be much more sensitive to an orientation of the substrate than are fibroblasts, which is surprising in view of the unpolarised form of so many of the reacting sarcoma cells. Secondly the distortion of the outgrowth continues after junction. An average of 70 per cent (determined by the Chalkley method [7]) of the clot surface is now occupied by fibroblasts in mouse muscle cultures, and an even higher proportion in chick heart cultures. This is the substratum on which the sarcoma cells are moving; and these fibroblasts are moreover themselves moving mainly in the opposite direction. It seems clear that the "attraction", far from being weakened by the change of substrate, must be stronger than ever. These points, while not invalidating a hypothesis of contact guidance, certainly suggest the possibility that the pattern of sarcoma cell outgrowth is moulded by substances diffusing from or into the explants of normal tissue. There are many indications in the literature that such diffusing substances can influence sarcoma cells [6, 10, 21].

Influences exerted after junction

When junction between the two outgrowths has been established, contact inhibition can come into play. There is no evidence that sarcoma cells are thus influenced when they encounter fibroblasts. According to our measurements no significant change occurs in the degree of distortion of the sarcoma outgrowth already existing before junction. In fact, as we have pointed out above, the sarcoma cells far from being inhibited are probably stimulated in movement. Since in addition the sarcoma cells after junction are randomly distributed on the fibroblasts, it may be concluded that there is no appreciable contact inhibition by fibroblasts of the sarcoma cells, with the proviso that there may be some elements in the heterogeneous sarcoma population to which this conclusion does not apply.

The behaviour of a fibroblast outgrowth on the other hand is markedly affected by junction with a second fibroblast outgrowth. This has previously been shown when two chick heart fibroblast outgrowths meet on a glass surface [4]. It has now been shown for mouse muscle when they meet chick

heart fibroblasts, on a plasma clot surface exactly as used in the tests of sarcoma against fibroblast. The effect is primarily on cell movement, which is obstructed by junction with another outgrowth so that the radial length of the outgrowth between the explants becomes significantly shorter than at the sides. The obstruction seems to check the movement of the entire sheet of chick heart fibroblasts, right back to the explant, from which fewer cells emerge on the obstructed side. The sheet of mouse muscle fibroblasts is checked, however, only peripherally within the duration of our experiment: cells continue to emigrate from the explant as before junction, and the density of population between the explants consequently rises fast, especially peripherally. This difference between the two types of behaviour is no doubt related to the density of population before junction, which is about three times higher amongst the chick heart than amongst the mouse muscle fibroblasts.

The argument that contact inhibition is responsible for this mutual obstruction of fibroblast outgrowth may now be set out as follows. The obstruction is not detectable before junction; indeed if anything there is enhanced outgrowth between the explants until junction is established. The data therefore provide no support for the existence of an inhibitory influence diffusing through the medium from the opposing explant; though they do not of course dispose of the possibility of such an influence with a short range of action. The peculiar distribution of the opposing cells after junction, with far fewer mutual overlaps than expected, indicates however that a local influence of cell upon cell is at work, and rules out the possibility that the obstruction is due to fibroblasts of one sort moving over fibroblasts of the other in contrary motion. We conclude therefore that contact inhibition operates between the two types of fibroblast.

There remains the curious point that the muscle fibroblasts are significantly obstructed by sarcoma cells but not vice versa. The interpretation of this is at present uncertain. There may be some interference with fibroblast movement by the overlying sarcoma cells. It also seems possible that a small proportion of the sarcoma cells, particularly those of the spindle form usually situated near to the sarcoma explant, may exercise contact inhibition on fibroblasts. Finally there is the possibility of a short-range diffusing inhibitor: the acid produced by tumours *in vitro* is a candidate [6, 11]. A deleterious effect of a tumour explant on nearby normal tissue was observed by Ludford and Barlow [16] but not by Jones [13].

Invasiveness

The work reported here points towards, though it certainly does not establish, two general conclusions. The first is that fibroblasts exercise contact inhibition on other fibroblasts even though the contacting cells come from different organs or from different species. This hypothesis has been severely tested since we have used two distinct kinds of fibroblasts, from different vertebrate classes. The second conclusion is that sarcoma cells in general are not, or are less, inhibited by contact with fibroblasts than are fibroblasts with each other. This hypothesis has not been severely tested, since the two kinds of sarcoma we have used are very alike, and moreover are very different from normal fibroblasts in form and behaviour. But that the failure of contact inhibition may apply to less anaplastic forms is suggested by reports of the intermingling of normal fibroblasts and those of Crocker 10 sarcoma in confronted cultures [6, 11].

Just as we suppose that contact inhibition keeps normal fibroblasts in place, so we suppose that its failure permits invasive behaviour. This point of view should be distinguished from that of Coman [8, 9], whose important work with carcinomata has led him to suggest that invasiveness is associated with a dimunution of adhesion between malignant cells, allowing them to break free and move away independently. Mutual adhesion is no doubt connected with contact inhibition [3]. We differ from Coman however in thinking (1) that separation of cells by loss of adhesion is not a prerequisite for movement, though it may well be for metastasis, and (2) that ability to undertake amoeboid movement is not enough to permit invasion unless freedom from contact inhibition goes with it.

SUMMARY

1. Mutual influences on outgrowth in tissue culture of mouse sarcomata (S 37 and Crocker), mouse muscle and chick heart fibroblasts have been investigated by the method of confronted cultures.

2. There are influences exerted before the outgrowths meet, causing cells to travel further towards an opposing explant than in other directions. In this respect the sarcoma cells are more affected by the fibroblasts than vice versa.

3. After their outgrowths have met, the two types of fibroblast obstruct each other's movement, since one cell rarely moves over another's surface (contact inhibition). No obstruction of sarcoma cell movement by fibroblasts

is detectable. Some obstruction of fibroblast movement by sarcoma outgrowth occurs.

4. The hypotheses are suggested that contact inhibition occurs between fibroblasts of different origin; and that malignancy involves a loss or diminution of this response to contact.

We should like to express our thanks to Professor J. Z. Young for reading this manuscript; and to the Nuffield Foundation for their support which made the work possible.

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AN EXPERIMENTAL ANALYSIS OF THE DEVELOPMENT OF THE SPINAL COLUMN

VI. ASPECTS OF CARTILAGE INDUCTION[1]

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Received March 4, 1957

In Needham's review [27] 26 embryonic induction systems are listed and since then many more have been described [7, 28]. Of these, the induction of neural tissue has been the most intensively studied and the most quoted in theories of embryonic induction. The experiments of Holtfreter and others (summarized in [20]) have revealed that a variety of tissues and agents simulate the action of the chorda-mesoderm so that one is dealing with a system activated by non-specific stimuli and hence difficult to analyze. In view of this difficulty in studying the induction of neural tissue and of the obscurity of inductive mechanisms in general, it seems desirable to study other induction systems, especially those characterized by specificity of interaction.

In preceding papers of this series (summarized in [1]) it was reported that the differentiation of chick somite cells into cartilage cells is dependent upon the inductive action of embryonic spinal cord or notochord. Somite cells reared in the absence of a cartilage inducing agent differentiate into mesenchyme and immature muscle cells.

The two major purposes of this paper are to (1) note the similarities and differences between the induction of cartilage in a population of chick somite cells and other induction systems, and (2) show that, although the spinal cord and notochord are relatively specific in their ability to induce cartilage, these two inducing agents have different modes of action.

[1] This investigation was supported by research grants from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service (B-493-C3) and the National Multiple Sclerosis Society.

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MATERIALS AND METHODS

The method of culturing was essentially that of Chen [4]. The desired tissue combinations were arranged on lens paper which floated on 1.8 ml of medium in an embryological watch glass. The medium consisted of Simm's salt solution, horse serum and 50 per cent, 11-day embryo extract (2:2:1). Since there was no difference in cartilage formation in cultures fed every 3rd day as compared with those fed once a week, the latter schedule was followed.

Individual somites, mesoderm-free spinal cord, and notochord were prepared as described previously [1]. Embryonic "mesodermalizing" agents [5, 30, 37] and devitalized bone and cartilage which induce ectopic cartilage in mature mammals were prepared by various methods [21, 23, 33].

For trans-filter experiments, a 2.0 mm hole was punched in the lenspaper and a strip of millipore filter (type TA, 20 μ thick with pore size 0.8 μ) placed across the hole. Under these conditions the central region of the filter, used for culturing, was in contact with the medium. The tissue was arranged on the filter and after 2–4 hours the filter with adhering tissue was removed from the lens paper and turned over. The second tissue was then placed on the other surface of the filter (Fig. 1). Additional details on filter and techniques for handling may be found in Grobstein [8].

In each experiment the issue was whether under the experimental conditions, somite cells would form cartilage. Each experimental series therefore consisted of three types of cultures, the experimental and two types of control cultures. The purpose of one set of controls was to demonstrate that somites would form cartilage in the presence of an inductor and consisted of somites plus spinal cord or notochord. The second control was to demonstrate that the somites would not form cartilage in the absence of an inductor and consisted of somites alone. Experimental and control cultures were set up from a common pool of stage 17–18 somites, spinal cord and notochord (stages of Hamburger and Hamilton [10]). Each experimental series was repeated, at least, on two different occasions. No fewer than 18 experimental cultures, plus the appropriate controls support the results reported in the text. Observations were made primarily on living cultures. In addition to the usual histological procedures with hematoxylin and alcoholic methylene blue, the Feulgen reaction was used for the detection of DNA, azure-B and aqueous thionine for

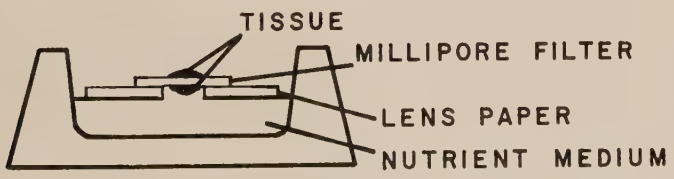

Fig. 1.—Diagrammatic cross-section of trans-filter induction experiments. The millipore filter, resting on the floating lens paper, is in contact with the nutrient medium. The reacting tissues and the inducing tissues are arranged on opposite sides of the filter. The culturing vessel is an embryological watch glass containing 1.8 ml of nutrient medium.

RNA and basophilia, and the periodic acid-Schiff reaction for polysaccharides. Bodian's reduced protargol was used to visualize nerve fibers and material in the millipore filter.

RESULTS

Specificity of cartilage induction.—Clusters of six to eight somites of stage 17–18 chick embryos fused and within 24 hours formed a circular mesenchymal sheet. Few additional changes were observed whether the cultures were maintained for one or two weeks. Of 259 such cultures (the cumulative number of controls) cartilage appeared in but two. With these two exceptions, histological examination of these cultures revealed only mesenchyme, immature muscle and occasional kidney tubules. Contrastingly, when similarly aged somites were clustered about a 0.5–0.7 mm length of spinal cord or notochord, sizeable masses of cartilage appeared by the fourth day of culture. Histological examination of the induced cartilage revealed the characteristic capsules and interstitial matrix. The matrix exhibited metachromasia with basic stains after five to six days of culture.

To test whether the cartilage inducing activity is a specific property of embryonic spinal cord and notochord, a variety of living and fixed tissues and inert objects were cultured with reactive somites. The size of the tissues and objects tested approximated that of the spinal cord or notochord routinely used. As shown in Table I, most of the agents tested were incapable of initiating chondrogenesis in embryonic somite cells.

It has been reported [5, 37] that alcohol fixed kidney and particularly, alcohol fixed guinea pig bone marrow [30] are potent embryonic "mesodermalizing" inductors. Exposure of salamander ectoderm to such alcohol fixed tissues resulted in the transformation of the ectoderm into mesodermal tissues. These same mesodermal inductors were incapable of causing cartilage formation in chick somite cells.

It has been suggested [19] that almost any agent provoking sublethal cytolysis will transform ectodermal cells into nerve cells and that neural inductors activate a neuralizing system that pre-exists within the ectodermal cells. To promote trauma in reacting somite cells, a heated needle was used to cauterize the central area of somite cultures after 24, 48, and 96 hours of incubation. The damaged area varied from approximately 100–1000 microns in diameter. In no instance did chondrogenesis take place.

Many investigators [6, 21, 23, 25, 26] have reported that living and devitalized cartilage and bone are capable of transforming embryonic fibroblasts *in vitro* and mature connective tissue cells *in vivo* into cartilage. It was

TABLE I

	Negative	Cartilage
Living tissues		
Notochord (3 days)	13	245
Spinal cord–ventral half (3 days)	12	240
Mesonephros (3 days)	35	7
Metanephros (12 days to post-hatching)	40	12
Adrenal (1 day post-hatching)	20	0
Bone (14 days, femur)	20	0
Bone marrow (guinea pig)	30	0
Cartilage (10, 12 days)	20	0
Liver (16, 17 days)	36	1
Lung (14, 17 days)	20	1
Muscle (14 days)	20	0
Nerve (15, 17 days, sciatic)	20	0
Pancreas (7, 9 days)	18	0
Small intestine (17 days)	21	0
Spinal cord–dorsal half (3 days)	20	0
Spinal ganglia (9 days)	20	0
Fixed tissues		
Bone (14 days, femur, alcohol fixed)	20	0
Bone (14 days, femur, frozen-thawed)	20	0
Bone marrow (guinea pig, alcohol fixed)	30	0
Cartilage (12 days, formalin fixed)	20	0
Cartilage (14 days, frozen-thawed)	20	0
Cartilage (14 days, alcohol fixed)	20	0
Liver (guinea pig, alcohol fixed)	20	0
Metanephros (guinea pig, alcohol fixed)	20	0
Muscle (12 days, formalin fixed)	22	0
Nerve (15, 17 days, sciatic, formalin fixed)	18	0
Notochord (3 days, formalin fixed)	21	0
Notochord (3 days, glycerinated)	22	0
Spinal cord (3 days, formalin fixed)	20	0
Others		
Glass beads	20	0
Wood	20	0
Cauterization	20	0
Somite controls (stages 17, 18, 19)	289	2

of interest to determine whether differentiated cartilage would induce reactive somite cells to form cartilage. Vertebral and rib cartilages from 10 and 12 day chick embryos were cultured with somite cells. There was no evidence that living cartilage could duplicate the inducing activity of notochord or spinal cord. Fixed and frozen-thawed nodules of cartilage were similarly inactive (see Table I).

Previous experiments with salamander and mouse material [9, 11, 17] stressed the localization of the cartilage promoting factor to the ventral half of the embryonic spinal cord. By culturing the alar or basal plate with reactive somites, it was clear that in the chick as well, the inducing activity is restricted primarily to the ventral half of the spinal cord. Spinal ganglia from 9 day chick embryos and peripheral nerves from 15 day chick embryos were inactive, indicating that the cartilage promoting activity is not a widespread property of embryonic nervous tissues.

Other than the spinal cord and notochord, only kidney, mesonephros or metanephros, induced cartilage. On a per cent basis, kidney was considerably less active than either notochord or spinal cord. Kidney tissue induced cartilage in 20 per cent of 94 cultures, whereas 85 per cent of over 500 cultures with spinal cord or notochord formed cartilage. However, the amount of cartilage in any single positive culture with kidney as inducer was of the same order of magnitude as that induced by spinal cord or notochord.

Some properties of the spinal cord-somite and notochord-somite interaction.— In any induction system the properties of the responding system appear to establish the nature of the reaction.

(*a*) That only somite cells can be transformed into cartilage by spinal cord and notochord was shown by the following experiments. Pieces of lateral mesoderm, liver and muscle, from 3 and 4 day embryos, comparable in size to that of the somites were cultured with spinal cord or notochord. In no instance did cartilage appear in these cultures. Similar results were reported for grafts in salamander embryos and larvae [14].

(*b*) It has been shown that amphibian ectoderm gradually loses its ability to respond to either neuralizing or lens inductors [18, 24]. That cultured somite cells also gradually fail to react to the cartilage promoting activity of spinal cord or notochord was shown by culturing somite cells for 24, 48, 72 and 96 hours before adding spinal cord or notochord. The yield of positive cases dropped from 80 per cent in somites pre-cultured for 24 hours before the addition of inductor, to 40, 0 and 0 per cent in the 2, 3 and 4 day series respectively. Whether the loss of reactivity is irreversible is still to be tested.

(*c*) Stage 17–18 somites consist largely of what histologically can only be

termed mesenchymal cells. These mesenchyme cells normally differentiate into the cartilages and connective tissues of trunk and dermis. Each somite of this age also possesses a small population of myoblasts synthesizing myosin and probably other muscle proteins and enzymes as well [16]. It was important therefore to determine whether this population of biochemically distinct cells would sort themselves into aggregates of similar types, comparable to those reported for amphibian cells [31]. In the event of a sorting out of cell types, the inducing activity of spinal cord or notochord might simply reside in its capacity to "attract" already determined procartilage cells. To test this possibility small patches of carbon were placed in a variety of locations on the mesenchymal sheet after 1, 2 or 3 days of culture. Following the initial radial expansion of the mesenchymal sheet there was no detectable displacement of the carbon markings during the entire culture period. Whether a given cell in the mesenchymal sheet forms cartilage appears to depend upon its immediate micro-environment rather than the movement of the cell to another location.

Differences in the inducing action of spinal cord and notochord.—There is a conspicuous difference in the distribution of cartilage induced by the spinal cord as compared with that induced by the notochord [12, 13]. In notochord-somite cultures the single piece of induced cartilage is always contiguous with the notochordal sheath. In contrast, cartilage induced by the spinal cord is always at some distance from the surface of the neural tissue and in culture often consists of several nodules. By altering the original distribution of somites the cartilage induced by the spinal cord may be made to form as much as 2.5 mm from the surface of the neural tissue [9]. These observations suggest that: (1) the spinal cord activity is mediated by a readily transmissible factor, whereas (2) the notochord activity is more local in its action and possibly is dependent upon the integrity of its sheath. The following experiments substantiate these views.

(*a*) When stage 18 notochord is cultured by itself it loses its sheath, its characteristic tubular configuration, and spreads as a sheet within 24 hours. These dispersed notochordal cells can be maintained in a healthy condition for over two weeks. Notochordal cells without a sheath were shown to be incapable of inducing cartilage by pre-culturing 0.5 mm pieces of notochord for 24 and 48 hours before adding the standard somitic mass. In no instance in this series did cartilage form. By contrast, spinal cord pre-cultured for as long as 4 days still induced cartilage when somites were added.

(*b*) If the notochordal sheath is the primary site of cartilage induction, insulating the sheath from reactive somite cells should block induction. This

was shown to be the case by surrounding the notochord by thin trips of 8-day trunk or limb muscle. The standard complement of somites were then added, the muscle serving as a barrier between somites and notochord. In these cultures the notochord differentiated in a normal manner, but did not induce cartilage in the somite cells.

Spinal cord does induce cartilage when similarly insulated from somite cells by strips of muscle. Apparently the transmissible cartilage promoting factor released by the spinal cord was neither trapped nor degraded by passage through the barrier of muscle tissue.

Trans-filter induction.—A neuralizing effect through a membrane [2] and passage through an agar film of the agent determining the direction of beat of epidermal cilia [32] has been reported. Interaction through a millipore filter in several other induction systems has also been demonstrated [7]. The following experiments demonstrate that (1) the cartilage promoting factor derived from the spinal cord passes through a millipore filter, whereas that from the notochord does not and (2) the passage of the spinal cord factor through the filter in effective quantities requires approximately 10 hours.

The standard number of somites was placed on one side of the millipore filter. Two to four hours later, the filter was turned over and the spinal cord placed above the somites (Fig. 1). Cartilage appeared in the somitic mesoderm 4 to 5 days later (Series 1, in Fig. 2).

There was no evidence for trans-filter activity by the notochord. When the notochord was placed on one side of the filter and somites on the other, the notochord did not maintain its sheath and no cartilage was induced even in cultures kept for over two weeks.

That the activity of the spinal cord is transmitted in effective amounts through the filter and for considerable distances through the mesenchyme,

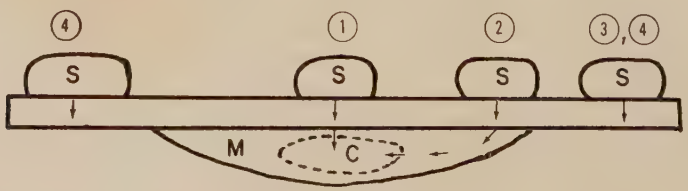

Fig. 2.—Diagrammatic view of the different types of trans-filter experiments. Spinal cord (*S*) on one side of the millipore filter, mesenchymal tissue (*M*) on the other side. *C* is the area where induced cartilage appears. With respect to the mesenchymal sheet, in series (1) the spinal cord is above the center, in series (2) the spinal cord is toward the periphery, in series (3) the spinal cord is lateral. In series (4) the augmented amount of spinal cord is also lateral to the mesenchymal sheet. Arrows represent the proposed direction and regions through which the cartilage promoting factor is transmitted.

but not through the ambient medium, is indicated by the following experiments. Spinal cord and somites were placed on opposite sides of the filter. In this series, however, the spinal cord did not lie directly over the center of the mesenchymal sheet but was so placed that after spreading of the somite cells the spinal cord either lay (1) over the periphery of, or (2) lateral to, the mesenchymal sheet underneath (Series 2 and 3, in Fig. 2). Cartilage did not form in cultures where the spinal cord was lateral to the mesenchymal sheet underneath (Series 3, in Fig. 2). Cartilage formed only in those cultures where the mesenchymal sheet spread beneath the spinal cord. Cartilage, in this series (Series 2, in Fig. 2) did not form directly beneath the spinal cord but toward the center of the mesenchymal sheet and appeared 12–24 hours later than in the standard trans-filter cultures. The location of the induced cartilage and the delay in its appearance in this series suggests that the inducing factor passed first through the filter, and then laterally through the mesenchyme, before acting on responsive somite cells. When the mesenchyme was not directly beneath the spinal cord, the factor emerging on the other side of the filter, apparently could not be carried in effective amounts through the medium to the reacting cells. It should be emphasized that the lateral displacement of the spinal cord from the mesenchymal sheet was often no greater than 50 microns. The arrows in Fig. 2 represent the proposed direction and regions through which the factor is transmitted.

The failure of the laterally displaced spinal cord to induce cartilage was probably due to failure of transmission in the ambient medium rather than dilution as suggested by the following experiments. As before, somites were explanted on the underside of the filter. But now instead of one 0.5 mm strip of spinal cord, four pieces each of 5.0 mm were placed lateral to the spread mesenchymal sheet (series 4 in Fig. 2). As in the previous series, cartilage failed to form in cultures unless some part of the mesenchyme came to lie directly beneath at least one area of the spinal cord. It is believed that if the spinal cord factor were readily diffusible in the liquid medium, augmenting the amount of spinal cord 40-fold should have induced cartilage.

An estimate of the time required for the passage of the cartilage promoting factor through the filter was obtained in the following manner. Somites were cultured on the filter for 2–4 hours. The filter was turned over and the standard piece of spinal cord placed over the somites. The spinal cord was then removed after varying periods of time. The results of these experiments are listed in Table II. It should be noted that the actual period of time for trans-filter passage of the cartilage factor in the different series is approximately 2 hours less than is recorded in Table II. For it requires at least 2 hours

Table II

Time somites exposed to spinal cord (hrs)	Number of cultures	
	Cartilage	No cartilage
2–3	0	15
5	0	25
10	12	28
16	18	20
24–48	33	6

for the spinal cord to simply adhere to the filter. The data therefore would indicate that within 10 hours the factor had passed through the filter in sufficient concentration to effect chondrogenesis. In these instances the amount of cartilage in the individual cultures was considerably less than that formed in the 24 to 48 hour series.

The histology and histochemistry of the filter between the interacting tissues. —Efforts to visualize material in the filter between spinal cord and mesenchyme were disappointing. With the Feulgen reaction for DNA the zone was negative. Tests for RNA and polysaccharide were likewise negative. Granules were seen in the filter with hematoxylin and much more prominently with Bodian's protargol method. With the protargol stained material, granules were observed to increase in number and depth of penetration in the filter as the culture aged. After 5 to 7 hours of incubation the granules penetrated approximately $\frac{1}{3}$ the width of the filter. These granules appear to be derived from the neural tissue. By 16 to 24 hours the granules are distributed throughout the width of the filter and by then some appear to be derived from the mesenchyme. Nerve fibers did not penetrate the filter to any great depth, nor did cytoplasmic processes span the filter.

These findings reveal very little about the nature of the material in the filter. The negative histochemical findings might be due to low concentration of material as well as its complete absence. Similarly the correlation between the penetration of the silver-positive granules and the time required for trans-filter induction while suggestive, requires further investigation. For example, peripheral nerves which we have found to be incapable of inducing cartilage, are known to produce in culture a silver-positive exudate [34].

DISCUSSION

In contrast to the induction of neural tissue from embryonic ectoderm, the transformation of somite cells into cartilage is mediated by a specific factor or factors. The factor is specific only in the sense that many other tissues or agents are not similarly active.

Nothing can be said at present as to the chemical nature of the cartilage promoting factor or even whether a single factor is common to spinal cord and notochord. If there is a common factor the difference in behavior of spinal cord and notochord could, in part, be explained by assuming: (1) the factor is present in lesser amounts in the notochord than in the spinal cord, or (2) the factor is not synthesized by the notochord, but is secondarily adsorbed from the ventral half of the spinal cord. Alternatively, the factor in the different inducing tissues may be biochemically distinct. The formation of cartilage in areas removed from the spinal cord or notochord (e.g. limbs, sternum, etc.) suggests a multiplicity of cartilage promoting substances.

In connection with the possibility of a multiplicity of cartilage promoting factors, it is of interest that living and fixed and frozen-thawed cartilage and bone failed to induce cartilage in somite cells. This contrasts with the activity of these agents in stimulating ectopic cartilage in adult connective tissue or from fibroblasts in tissue culture [6, 21, 23, 25, 26, 33]. The consensus of recent workers on the induction of ectopic cartilage is that the chondrogenetic stimulus is non-specific. Living or devitalized bone and cartilage, alcohol, and mechanical trauma are thought to stimulate chondrogenesis as a consequence of provoking and inflammatory response [21, 33]. The inability of these agents to induce chondrogenesis in somite cells may be attributed to any of the following: (1) the absence of the inflammatory response *in vitro*, or (2) the difference in reactivity of the somite cell as compared to the mature connective tissue or fibroblast cell, or (3) the fact that even adult connective tissue cells require several weeks from the time of stimulation to form cartilage.

The formation of cartilage in kidney cultures may be explained by the kidney mesenchyme itself transforming into cartilage. Recent unpublished work has shown that embryonic kidney by itself as well as kidney plus reactive somites, will produce cartilage.

Little is known of the passage of material through embryonic tissues. India ink particles picked up by entoderm cells eventually are passed on to epidermal cells [29]. Material released by gonads of the toad affect tissues within a radius of several hundred micra [36]. Likewise, the transmissible

properties of the spinal cord cartilage promoting factor are not readily interpreted. It appears to pass through a mesenchymal or muscle tissue continuum. It passes less readily, if at all, through the liquid medium. Whether the factor passes directly through the millipore filter or has certain requirements for transmission is unknown. It has been suggested that the pores of the filter may contain extracellular ground substances [8]. Thus the cartilage factor may be transmitted through the membrane in this extracellular continuum. Possibly the interaction in some embryonic induction systems is to be sought along these lines—molecules not readily soluble in aqueous solutions but capable of limited diffusion in the complex continuum between cells [3, 7] or of being passed along from cell to cell [35].

The variability in response following different exposure to the spinal cord requires some comments. On the basis of glucoronic acid determinations it has been found [22] that the time of appearance and amount of chondroitin sulphate synthesized in individual notochord–somite cultures, varies considerably. Probably this variability is a function of the condition of the somites at the time of explantation [1], so that in the trans-filter experiments one culture requires less of the cartilage factor than another culture, presumably depending on the *in vivo* exposure of the somites to the cartilage factor prior to explantation. Pertinent to this is the fact that many cultures of stage 20 somites by themselves will differentiate into cartilage after four days of culturing. It is suggestive that stage 20 somites are approximately six to eight hours older than stage 17–18 somites—a period of time approximating that required for trans-filter exposure to be effective in many cultures. Taken together these observations would suggest that stage 17–18 somites: (1) have been exposed to a sub-threshold dose of cartilage promoting factor at the time of explantation, (2) will self-differentiate into cartilage when exposed to the cartilage factor for an additional eight to ten hours either *in vivo* or *in vitro*, and (3) require a period of three days from the time of exposure to the cartilage factor to differentiate into cartilage cells.

SUMMARY

Both the embryonic spinal cord and notochord induce the formation of cartilage in a population of chick somite cells. This inducing capacity is relatively specific—most other living and fixed tissues, including living and fixed cartilage, are ineffective. The cartilage promoting activity of the spinal cord appears to be mediated by a factor transmissible through mesenchymal or muscle tissue and through a millipore filter. The cartilage promoting

activity of the notochord acts more locally and appears to be associated with the notochordal sheath.

The time for the passage of the spinal cord cartilage promoting factor through the millipore filter is approximately eight hours.

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A COMPARISON OF SEVERAL DEOXYRIBONUCLEASES OF TYPE II[1]

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Received March 6, 1957

Although deoxyribonucleases (DNases) have been encountered in a great number of different biological materials, it is only within the past few years that attempts have been made to distinguish one such enzyme from another. In 1949 Zamenhof and Chargaff [25] showed that the DNase of bovine pancreas (DNase I) [7] and of yeast, both of which require magnesium, differ in pH optimum and other respects. DNase II [7] which does not require magnesium and shows greatest activity at pH below neutrality, was first discovered [5, 15] in bovine thymus and spleen. In contrast to the extracellular pancreatic and streptococcal [16] enzymes—which appeared to be similar on the basis of their common magnesium requirement, and might be assigned digestive functions—DNase II (intracellular DNase of Webb) [24] was found in nuclei [4, 22, 1] and in cytoplasmic particles [4] of mammalian cells and has been likened to cathepsin [15, 22]. Some investigators [22, 1] have suggested a role for DNase II in the metabolism of deoxyribonucleic acid, such that it would play an essential part in cell proliferation.

Evidence has been accumulating that there are many magnesium-activated DNases, which may be differentiated by the use of specific inhibitors [25, 2, 12], or by studies of the "core" [3] remaining after DNase action. Recently [6], a bacterial enzyme has been shown to differ from both DNase I and II in various ways, including cation requirement and pH optimum. Thus it seems that there is great variety among the deoxyribonucleases, and that it would not be surprising if "DNase II" should prove to be heterogeneous.

We wish to present certain findings concerning enzymes of the DNase II type produced by a cyclostome, by a protozoan, and by two vertebrate exocrine glands, which suggest that DNase II represents a class of enzymes, which differ not only in various chemical properties, but probably also in function.

[1] Supported by a Research Grant, C 2128, from the National Institute of Health, U.S. Public Health Service.

MATERIALS AND METHODS

DNase assay.—Highly polymerized sodium deoxyribonucleate was prepared by the method of Kay *et al.* [11] and was used in all assays at a final concentration of 1 mg/ml. The reaction mixtures contained 0.01 *M* sodium citrate (to inhibit magnesium-requiring enzymes) and 0.1 *M* acetate buffer at the optimum pH (5.0–6.2) of the enzyme being studied. One part of enzyme solution, suitably diluted, was added to seven parts of the substrate-buffer mixture. A portion of the mixture was then drawn into a microviscometer[1] and the flow time of the mixture determined at intervals, while digestion proceeded in a 37°C water bath. The principle of calculation of units of activity from the rate of decline of the flow time was essentially that of Laskowski and Seidel [13]. The flow times, minus the flow time of water in the same viscometer, were plotted against incubation time on semi-logarithmic paper and the slope of the straight line portion of the plot was used for calculating units of enzyme activity per ml of the enzyme solution. If there was a fall of 0.001 per minute in the logarithm of the corrected flow time, the activity was expressed as one unit per ml. For some purposes a time-saving semi-quantitive method similar to one described by McCarty [17] was used for following the disappearance of highly polymerized substrate. Reaction mixtures were prepared as for the viscometric method, but were incubated in a test tube rather than a viscometer, and at intervals a drop of the mixture was allowed to fall into 90 per cent acetone, till the formation of a web-like fibrous precipitate failed to take place. Allowing for the uncertainty inherent in determining the exact time required to reach this end-point, the reciprocal of the required time was found to be proportional to the viscometric units of a given enzyme preparation.

Sources of enzyme.—A culture of the ciliated protozoan, *Tetrahymena pyriformis* strain WH was kindly supplied by Dr. George Kidder. For DNase production it was grown with aeration in a medium containing 0.13 g $MgCl_2$, 1.3 g Na_2HPO_4, 0.65 g KH_2PO_4, 0.13 g DL tryptophan, 4.0 g glucose, 4.0 g Difco Bacto Yeast Extract and 6.6 g Difco Bacto Casamino Acids per liter, at room temperature for 7 to 9 days, and the resulting dense cultures (about 5×10^5 ciliates per ml) were filtered through fluted Whatman #50 paper.

Dried venom of *Crotalus adamanteus* was purchased from the Ross Allen Reptile Institute, Silver Springs, Florida.

Live specimens of the lamprey, *Petromyzon marinus*, were obtained through the courtesy of the Wisconsin Bureau of Fish and Wildlife, and the tissues were used immediately after decapitation. Other crude enzyme sources were obtained, refrigerated, from local wholesale food suppliers and were used as soon as possible. For all tissues studied, one gram was homogenized with ten ml of distilled water (or of 0.9 per cent NaCl, in the case of spleen) in a Waring Blendor, and the clear supernanant was used after centrifugation.

Partially purified [14] DNase II of calf thymus was a gift from M. Laskowski.

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[1] Bore 0.45 mm, length 14 cm, flow time 13–20 seconds, single bulb. Otto R. Greiner Co., 221–223 High St., Newark 2, New Jersey.

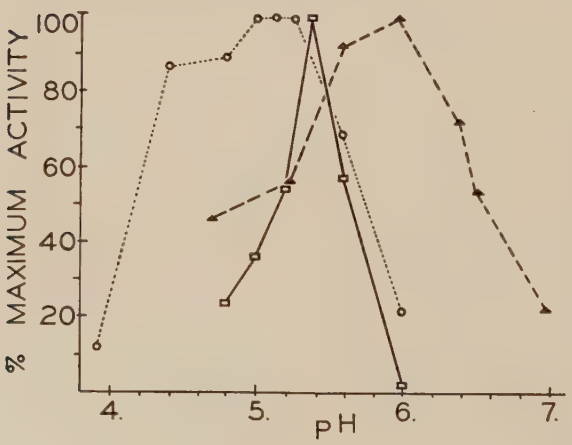
RESULTS

Extracellular DNase II

DNase II of Tetrahymena.—This enzyme appears to be the same as that which was studied in homogenates of *Tetrahymena* cells by Eichel and Roth [9]. Our preliminary studies indicated that, while the concentration of the enzyme was higher intracellularly than extracellularly, by far the greatest part of the DNase of the whole culture was in the culture filtrate. The enzyme was stable overnight at room temperature in culture filtrates. It could be concentrated, with some loss, by saturating the filtrate with $(NH_4)_2SO_4$ and collecting the very fine precipitate by filtration through Whatman #50 filter paper, or by centrifugation at 12,000 rpm for 30 minutes. After dialysis the enzyme was then found to be relatively unstable. The pH optimum was 6.2 (see Fig. 1).

DNase II of rattlesnake venom.—Venom of *C. adamanteus* was one of the most potent sources of DNase II that was studied (Table I). The pH-activity curve was quite similar to that of the mammalian DNases (Fig. 2). The enzyme was fairly stable in the dried venom, the loss of activity in two years at room temperature being 70 per cent.

DNase II of cow's milk.—Preliminary investigation of mammalian secretions indicated that human saliva, bull semen (from the epididymis), and ox bile



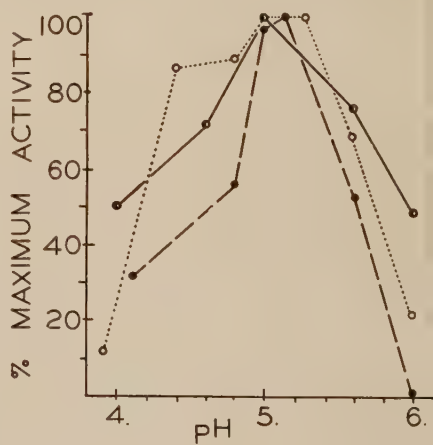


Fig. 1 *(left).*—pH-activity curves of various DNase II preparations from phylogenetically unrelated animals. ○·····○, cows' milk; ▭———▭, lamprey muscle; △ – – – △, *Tetrahymena* culture filtrate.

Fig. 2 *(right).*—pH-activity curves of various DNase II preparations from higher vertebrates. ○·····○, cows' milk; ◐———◐, calf thymus; ● – – – ●, rattlesnake venom.

TABLE I

DNase II activity of various crude sources.

Source	Quantity containing 1000 viscometric units measured at optimum pH
Tetrahymena culture medium . . .	1–2 ml
Dried venom, *Crotalus adamanteus* .	0.017 g
Cow milk	100 ml or 10 minutes' production
Cow plasma	1,000 ml
Bovine spleen	0.3 g
Bovine liver	10.0 g
Lamprey liver	0.03 g
Lamprey muscle	0.15 g

had little or no DNase II. In raw cow's milk, however, DNase could be detected readily (Table I). Although the DNase II activity of milk was lower than that in any of the other sources cited in Table I except plasma, the minimum average production at this dairy farm is three gallons per day per cow, so that the DNase II secreted in thirty minutes would be equal to the amount extractable from one gram of cow spleen, or thirty grams of cow liver. The site of origin of this enzyme would appear to be the mammary epithelium, rather than plasma, inasmuch as plasma has at most very little DNase II activity (Table I), and mixture experiments showed that this is not due to the presence in plasma of an inhibitor which is effective against the DNase II of milk. Therefore (unless there is simply a slower "turnover" of DNase II in milk than within various tissue cells) it would appear that the mammary epithelial cells are unusually active producers of DNase II.

It was impossible to obtain a pH activity curve for the DNase II in crude milk because of precipitation of casein at pH 4.5 or below. After centrifugation at 25,000 rpm for thirty minutes[1] a sediment and a floating layer of fat were removed. The pH of the supernatant was adjusted to 4.9 with 1 *M* acetic acid. A precipitate was removed by centrifugation at 14,000 rpm for 15 minutes. The supernatant was adjusted to pH 5.5 with 5.0 *N* NaOH and was fractionated with $(NH_4)_2SO_4$, at 20, 50 and 80 per cent saturation. The

[1] A Spinco ultracentrifuge was used through the courtesy of M. Laskowski.

Table II

Exp.	Electrophoresis of DNases pH	Hours	Field strength v/cm	Source of enzyme, and velocity (−, toward cathode), 100 × cm/hr/field strength Calf thymus	Lamprey muscle
1	7.7	1.25	21	0	6
2	5.7	12	9	−1.5	1
3	4.2	5	9	−3.5	0
4	4.1	12	9	−5	−2

Buffers for electrophoresis: 0.05 *M* veronal for #1; 0.02 *M* succinate for #2; 0.025 *M* citrate for #3; 0.05 *M* acetate for #4. Temperature for all runs, 2–5°C.

material which precipitated between 50 and 80 per cent saturation was dissolved in a minimum quantity of water and dialyzed against distilled water. The pH was then adjusted to 2.5 and a 50 to 80 per cent saturated ammonium sulfate fraction was prepared similarly. The pH optimum for activity could then be determined and was found to be the same as that of other mammalian DNase II (Fig. 2).

"*Intracellular*" *DNase II*

DNase II of lamprey tissues.—In preliminary experiments, extracts of various mammal, bird, reptile and teleost tissues were compared, with results generally in agreement with those of previous workers [22, 1]. Most organs, especially those of mammals, were rather poor sources of DNase II. In animals having a spleen, this was always the best source. However, it was unexpectedly found that the sea lamprey, *Petromyzon marinus*, provided very active extracts, and this species was selected for more intensive examination. Liver was the most potent source of enzyme (the lamprey has no spleen) and skeletal muscle the least potent (Table I) while values for intestine, kidneys and gonads were intermediate. The optimum pH with either liver or muscle extracts was 5.4 (Fig. 1), the activity falling off steeply on either side. The muscle extract was concentrated by precipitation at room temperature with saturated ammonium sulfate at pH 5.9 and dialyzed against distilled water. It was then found possible to compare this enzyme with calf thymus DNase II (also concentrated by precipitation and dialyzed) [14] by means of paper electrophoresis. An open-strip apparatus was used as recommended by McDonald [18]. Conditions of pH, voltage, etc. are presented in Table II.

For localization of the enzyme-containing spots, transverse segments of the paper strips were cut at 0.5–2.0 cm intervals and were incubated in 5 ml of the ordinary buffered substrate mixture at the optimum pH of the individual enzyme, and the progress of the digestion was followed by the acetone precipitation method. When the reaction mixtures bathing one or two of the segments showed no fiber formation, more accurate localization was obtained by determining the viscosity of all the reaction mixtures. Of the two enzymes, lamprey DNase II was the more acidic protein, showing an isoelectric point at pH 4.2 in these experiments. The lamprey muscle enzyme, extracted with water from a spot produced as in Exp. 1, Table II, showed the same pH-activity curve as crude muscle extract.

DISCUSSION

It is possible that the DNase activity observed in snake venoms by Delezenne and Morel [8] and by Taborda *et al.* [23], in *Tetrahymena* homogenates by Eichel and Roth [9, 21], or in milk by Greenstein and Jenrette [10] might have been due, in whole or in part, to DNase II. In none of these investigations was the addition of magnesium shown to be essential for activity. No data seem to have been obtained, in any case, concerning pH optima.

With many tissues and secretions, the situation may be complicated by the presence of more than one DNase [1, 7]. We have found that the DNase activity of raw milk, under optimal conditions for action of DNase I [13], was about half that obtained under conditions optimal for the DNase II. With the venom of *Bothrops jararaca*, Taborda *et al.* [23] measuring DNase activity manometrically at pH 7.5, found responses to addition of various cations incompatible with the presence of either DNase II or DNase I, alone. The question of the presence in milk and snake venom of DNases other than DNase II remains unsettled.

On phylogenetic grounds, and from the dissimilarity of the cells of origin, as well as from the data we have obtained, we are inclined to consider the DNases II of *Bos*, *Crotalus*, *Petromyzon*, and *Tetrahymena* as unrelated enzymes which happen to resemble each other in not requiring magnesium, and in having more or less acid pH optima.

It might be argued that the DNase of *Tetrahymena* is released only as the organisms disintegrate. This would be an alternative to our interpretation, that it is secreted into the culture medium. However, cells from our cultures in the stationary phase, when the highest levels of DNase are reached, were rarely observed to undergo fission, so that conditions seemed to be comparable

to those obtaining in Ormsbee's [20] investigations. Ormsbee concluded that in the stationary phase of *Tetrahymena* cultures the rate of death and replacement of the protozoa was negligible.

Thus our data would indicate that DNase II appears not only intracellularly (therefore measurable only in tissue extracts), but also extracellularly in bodily secretions such as milk and venom. This contradicts the suggestions of several previous authors [1, 4, 7, 15, 24] that DNase II is intracellular. However, it is possible that the extracellular DNase II has no function at all, and simply appears as the result of some sort of accident. The apocrine method of secretion of the mammary gland might favor this, since part of the cell disintegrates during milk production. However, since such relatively large quantities of DNase II appear in some secretions, this would require that the concentration of DNase II within cells has been grossly underestimated because of incomplete extraction of the enzyme from nuclei, mitochondria, or other organelles. Several investigators have attempted to prevent such an error by incubating tissue homogenates or particle preparations in substrate solutions, and determining the rate of release of small split products, rather than reduction of viscosity. However, under such circumstances, the limiting factor may still be the accessibility of the enzyme to the substrate [19] rather than the total amount of enzyme.

SUMMARY

Deoxyribonuclease II is a class of enzymes which have pH optima below neutrality, and which do not require activation by divalent cations. Such enzymes are found not only in various mammal tissues, but also in milk, snake venom, cyclostome tissues, and culture filtrates of the ciliated protozoan, *Tetrahymena pyriformis*. Deoxyribonuclease II of calf thymus, lamprey muscle, and *Tetrahymena* have different pH optima. The calf and lamprey enzymes behave differently in paper electrophoresis. Enzymes of this class apparently differ in chemical nature and also in their function.

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THE LOCALIZATION OF PHOSPHORUS AND THE SITE OF CALCIUM BINDING IN THE YOLK PROTEIN OF THE FROG'S EGG

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Received March 10, 1957.

Recently Barth and Barth [1] achieved a partial fractionation of vitellin, the main yolk protein of frog eggs (*Rana pipiens*), by precipitating one fraction at pH 8.8 and a much smaller fraction at pH 3.0–3.5. Incubation with pigment granules, which possess phosphoprotein phosphatase activity, suggested that the smaller fraction which precipitated at the acid pH, contained most of the protein phosphorus of the complex. These authors believe that the action of phosphoprotein phosphatase is to split phosphate from this smaller component and thus release the larger component from the water-insoluble complex in a soluble form.

Schjeide, Levi and Flickinger [7], utilizing the ultracentrifuge, also found vitellin to consist of at least two components (as did Gross and Gilbert [4]) but did not establish whether these were similar to the electrophoretic components reported by Barth and Barth [1]. It was our purpose to determine if one of the two ultracentrifugal components of vitellin likewise contained most of the protein phosphorus and also to further clarify the role of the calcium ion in solubilizing vitellin by ascertaining if either of the two components could bind calcium. Both of these objectives demand a means of separating the two components. In our case this was accomplished by ultracentrifugation within the partition cell [6] designed for the preparatory ultracentrifuge.

EXPERIMENTAL

The details of the method of preparation of vitellin and livetin from frog eggs and embryos are given by Flickinger [3]; the method is essentially one of dissolving washed yolk platelets in 10 per cent NaCl, dializing against distilled water (2°C) to precipitate vitellin, and water-washing this precipitate. Livetin is the water soluble supernatant remaining upon dialysis.

TABLE I

Fraction	Phosphorus/nitrogen ratios		
6S Component of vitellin dissolved in 10% NaCl	0.250	0.159	0.183
6S + 11S fraction of vitellin dissolved in 10% NaCl	0.053	0.043	0.064

Some of the vitellin was dissolved in 10 per cent NaCl and centrifuged at approximately 2500 *g* to remove undissolved material and then subjected to ultracentrifugation at 75,000 *g* for 9 hours in a Spinco preparatory ultracentrifuge using the partition cells. A portion of the lighter component (6S) was isolated in the top segments of this cell while the bottom segments contained both the light and heavy components (6S + 11S).

Phosphorus/nitrogen ratios were ascertained for the isolated 6S and 6S + 11S fractions: both fractions were extracted with cold 10 per cent trichloracetic acid, boiling alcohol-ether and hot 5 per cent trichloracetic acid in order to remove acid soluble materials, lipids, and nucleic acids. The acid-insoluble residues were washed and suspended in water and samples were removed from each for total nitrogen estimations [8]. The remainder of the samples were diluted with equal volumes of 4 per cent NaOH and phosphoprotein phosphorus was hydrolized by boiling for ten minutes; inorganic phosphorus was then determined by the Fiske–Subbarow method [2]. These phosphorus/nitrogen ratios are reported in Table I. The higher P/N ratio of the smaller 6S component indicates that it is the phosphoprotein. Phosphorus analyses therefore allow the assumption that the smaller electrophoretic and ultracentrifugal components are essentially similar and supports the idea of Barth and Barth [1] that phosphoprotein phosphatase action removes phosphate from the smaller electrophoretic component thus releasing the larger component from the water-insoluble 6S + 11S complex in a water soluble state. The water-soluble protein sedimenting at 11S is apparently equivalent to one of the components of livetin.

Although the P/N ratios show the smaller 6S component to be the phosphoprotein, there is much more phosphoprotein phosphorus present in the larger 6S + 11S fraction. The high level of phosphoprotein phosphorus in 6S + 11S is attributable to 6S sedimenting into the bottom segment along with 11S material.

Barth and Barth [1] report that at pH 4.7 a large component of vitellin

can be precipitated by sodium adenosinetriphosphate or by isolated phosphoprotein. Some of our experiments also demonstrate marked affinity of phosphoprotein for other molecules. If some isolated phosphoprotein (6S) is mixed with livetin (11S) in distilled water, a precipitate occurs which has some of the characteristics of vitellin (6S + 11S); e.g., this precipitate could be dissolved by 5 per cent NaCl, or lower concentrations of $CaCl_2$, and analytical ultracentrifugation of the NaCl dissolved precipitate provided patterns similar to that of native vitellin (6S + 11S). It is believed that the action of the calcium is that of combining with some of the 6S protein phosphate groups thus rendering them inaccessible for combination with polar groups of the 11S component and hence splitting and solublizing the 6S + 11S complex. Five per cent sodium chloride apparently solubilized the complex primarily by virtue of its polar properties at this concentration.

In order to test the idea that the protein phosphate groups of frog yolk protein bind calcium, it was decided to add $CaCl_2$ to whole yolk, vitellin and livetin preparations, and then to ultracentrifuge using the partition cells, and to assay for calcium in the separate segments. Thus accumulation of calcium in any of the lower segments, which was above the level of unbound calcium in the top segment, would indicate that there was active adsorption of calcium by that fraction. Phosphoprotein phosphate assays could then be used to ascertain if this fraction was high in protein phosphate groups.

The fractions were separated from the segments and the protein was precipitated and the bound calcium released into solution by adding trichloracetic acid to a 10 per cent concentration. In the case of ovarian vitellin little or no protein was found in the top segment (disc 1), a small amount of the phosphoprotein (6S) was found in segments 2 and 3 together, and a large amount of 6S + 11S is precipitated from the bottom segment (disc 4). The TCA supernates were subjected to the colorimetric calcium method of Roe and Kahn [5] and these results, based upon equal sample volumes, are given in Table II.

Calcium assays upon the separated fractions of ovarian yolk and vitellin clearly showed that most of the calcium appeared in segment 4, which contains the bulk of the protein (6S + 11S), less calcium was in segments 2 and 3, and still less in segment 1 where very little protein was present. Although estimation of the protein phosphate revealed that more of the phosphoprotein was present in the 6S + 11S complex in segment 4, than in discs 2 and 3 (6S alone), still some doubt existed as to whether the calcium was being bound by protein phosphate groups. It was possible that the bulk of the sedimenting protein carried calcium with it in some less specific

TABLE II

Relative amounts of calcium in the segments based upon similar volumes.[a]

Fraction	Experiment number 1	2	3	4
Ovarian vitellin				
Mg/cc of calcium added	1	1	1	1
Partition cell segment 1	25	26	25	36
2 & 3	30	40	40	39
4	38	51.8	52	63
Ovarian yolk				
Mg/cc of calcium added	0	1		
Partition cell segment 1	6	59		
2 & 3	12	49		
4	17	98		
Livetin				
Mg/cc of calcium added	0.5	0.5	0.5	
Partition cell segment 1	70	31	51	
2 & 3	26	38	58	
4	39	34	41	

[a] These values may only be compared within a given experiment since the concentrations of protein in the various fractions were different.

manner. However, this possibility was dispelled when livetin (the water-soluble yolk protein) prepared from larvae was ultracentrifuged with calcium added. In this case most of the protein again was sedimented to the bottom segment 4, but the calcium level here was no greater than the top segment (no protein), and where there was slightly more calcium in segments 2 and 3 there was less than 1/10 the amount of protein as in disc 4 (Table II). There was apparently no binding of calcium when livetin, which has no phosphoprotein (6S) component [7], was ultracentrifuged. This makes it appear that there was selective binding of calcium by the smaller phosphoprotein component (6S) of the 6S + 11S vitellin complex and that the greater mass of protein was not merely adsorbing calcium in a non-specific manner.

SUMMARY

1. A partial separation of the two components of vitellin, the yolk phosphoprotein of frog eggs, was obtained utilizing an ultracentrifugal technique.

Phosphorus/nitrogen ratios revealed that the slower sedimenting component of vitellin is the phosphoprotein.

2. The addition of calcium chloride to vitellin, and ultracentrifugal separation of the components, demonstrated that the phosphoprotein component can selectively bind calcium.

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Experimental Cell Research **13**, *317–332 (1957)*

REACTIONS OF *STENTOR COERULEUS* TO CERTAIN SUBSTANCES ADDED TO THE MEDIUM[1]

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Received March 21, 1957

A SERIES of simple tests showed that solutions of various salts, sugars, and other chemicals have highly selective effects on cytoplasmic differentiations in the fresh water ciliate *Stentor coeruleus*. These effects occur promptly and are usually followed by complete recovery of the cell on return to normal medium. Hence the chemical treatments herein described have a twofold interest: (1) they provide an easy means for selective operations on various parts of the cell, and (2) they have a bearing on the nature of cytoplasmic differentiation.

In 1909 Prowazek [13] reported briefly that a 0.5 per cent solution of table salt caused *S. coeruleus* to shed a halo of its blue-green pigment and to cast off the peristome. Full pigmentation was later restored and the peristomial band of ciliary membranelles was regenerated. This observation has now been confirmed and is considerably extended in the present study.

Later investigators studied the effects of salts and sugars on various species of *Stentor* from a physiological standpoint. Child [3] observed a polar disintegration of *S. coeruleus* in KCN as substantiating his theory of metabolic gradients. In an extensive study Merton [9, 10, 11] compared various anions and cations and two sugars with regard to their capacity to induce reversal of ciliary beating, and he also found that potassium produced partial anaesthesia of the myonemes. Heilbrunn [6] reported that mono- and bivalent ions had reciprocal effects on the viscosity of the endoplasm, as well as that Ca is necessary for the surface precipitation reaction in *Stentor*. Weisz [19] found that Janus green caused sloughing of pigment and of a cortical outer layer and that the pigment granules had a selective affinity for this stain. Recently Sleigh [14] investigated the effects of $MgCl_2$ and other substances on the beating and coordination of peristomial membranelles in *S. polymorphus*.

Other workers have studied the morphological effects of salts and/or sugars on ciliates other than *Stentor*. In particular may be mentioned Browning's [2] demonstration of an antagonistic effect between Ca salts and osmotic pressure due to sucrose, resulting in abnormal forms of *Tetrahymena geleii* attributed to inhibition of cleavage and random cell elongation; and Fauré-Fremiet's [4] report on the production of microstomatous forms of *Leucophrys* (*Tetrahymena*) *patula* by LiCl.

[1] This project was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

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MATERIALS AND METHODS

Five strains of *S. coeruleus* from different localities were drawn upon in this study. The general response of individuals from all these stocks was the same. Stentors were grown in filtered lake water with colorless flagellates and rotifers as food organisms, using skimmed milk as the basic nutrient [15]. This method provided healthy cultures which could be kept indefinitely. A few confirmatory tests were made on two other species (*S. roeseli* and *polymorphus*) cultured in the same manner.

Solutions were prepared by dissolving crystallized chemicals in the Millipore-filtered Loomis Lake water which was used in the stock cultures. This is a "young" bog lake on an ocean-beach peninsula, the waters of which could be expected to be "soft". Distilled water was not used because, as Merton [11] found, pure water is itself toxic to stentors. Like Merton I also found that there was considerable variation in details in the response of stentors, which depended on the condition of the culture and especially, it seemed, on the nutritive state of the organisms. Therefore one cannot assert that a specific concentration will always produce the same reaction. For the short-term tests, I started with a concentration which would kill the stentors within an hour but not sooner than 15 minutes; and for long-term experiments a graded series was used within the range of which would be found concentrations in which the stentors could survive for a week or 10 days -the survival time of control animals isolated without feeding in caster dishes of lake water. The results given were typical for animals concentrated into a covered dish (50 ml) and left for 1 to 3 days before using. Observing the stentors under a dissecting microscope at magnifications of 150× with reflected light from an embryological lamp revealed specific reactions in the cortical differentiations of the cell. All experiments were conducted at room temperature (60°–70°F).

RESULTS

Shedding of the peristome and disintegration of other ectoplasmic structures of S. coeruleus *produced by brief exposure to lethal dilutions of sea water*

Stentors were subjected to a solution of Willapa Bay water mixed with the water used in culturing in proportions to give a strength of about 25 per cent standard sea water, or a salinity of about 8 parts per thousand.

The initial response was that (*a*) often but not always the stentors sloughed a soft halo of their blue-green pigment but most of the pigmentation remained in place in the pigment granules of the ectoplasm. (*b*) Next, the animals swam vigorously backward for 1–2 minutes with the peristomial membranelles stopped and held forward while the stroke of the body cilia was reversed, followed later by more normal behavior with forward swimming. (*c*) The ciliary components of the membranelles then seemed to spread apart, giving a fimbriated appearance to the peristomial band (as in Fig. 9). (*d*) Then the animals suddenly contracted into a sphere as if salts had finally

penetrated into the cell causing excessive irritation. This reaction proved to be a useful signal of the next event: (*e*) shedding of the whole peristome intact, including all the membranelles and whatever may hold them together in a band, those lining the cytopharynx being the last to be set free (Fig. 1 *a*, and as in Figs. 9, 10, 11, 12). (*f*) Usually the holdfast at the posterior end of the cell next disintegrated. (*g*) Finally, the ectoplasmic structure of the cell

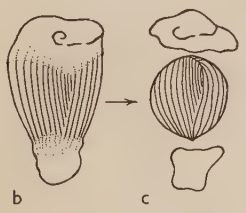


Fig. 1.

Fig. 2.

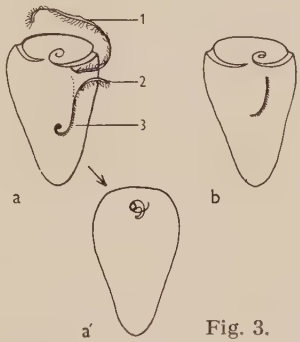


Fig. 3.

Fig. 1.—Reaction of *S. coeruleus* to lethal concentration of sea water. (*a*) Peristome of ciliary membranelles fimbriated and being shed but still attached in the oral region. (*b*) Holdfast disintegrated and ectoplasmic structure being disintegrated in waves from the anterior and posterior ends of the cell. (*c*) Recovery of such an animal, disintegrated parts sloughed and central, intact portion healing over to regenerate.

Fig. 2.—Reaction of *S. coeruleus* to Holtfreter's solution. Membranellar band is shed first in the region of the cytopharynx and/or along the border of the buccal cavity (×).

Fig. 3.—Reaction of reorganizing *S. coeruleus* with oral primordium to lethal concentration of sea water. (*a*) Original peristome first shed (1), then advanced primordium sloughed beginning at the anterior end (2). (*a'*) Cell developed only a cytopharynx from the remaining posterior portion of the primordium (3). (*b*) Early primordium unaffected long after original peristome has been shed.

body as manifest in the pigment stripes began to disintegrate in 2 advancing waves, first from the anterior and secondarily from the posterior end (Fig. 1 *b*). Body cilia no longer beat where this occurred, the kineties between the stripes presumably also disintegrating. (*h*) The extremity from which the animals could recover was reached when disintegration had progressed until only about half of the original ectoplasm remained intact around the middle of the cell. In such recoveries the affected ends of the cell were sloughed on return to normal culture medium. The mid-region then healed over and regenerated a normal stentor (Fig. 1 *c*).

No exceptions to this sequence of events were observed among the 34 specimens individually tested. The reaction could be stopped at any stage by quickly transferring the animals to normal medium. Increasing the concentration of the sea water merely accelerated the sequential destruction

of cell differentiation; and when disintegration of the anterior ectoplasm followed rapidly after lifting of the peristome, the whole anterior end of the cell was often sloughed, mouth-parts and the adoral field encompassed by the peristome also being pinched off.

Decreasing the concentration or the time of exposure showed that disintegration and shedding of adoral membranelles was an all-or-none phenomenon. Although only part of the adoral band may be shed in minimal treatments, the sloughing of any section of membranelles was complete down to and including the band which holds them together and there were no instances of partial damage to membranelles.

The most arresting feature in the response of stentor to sea water was therefore the selective effect on a hierarchy of cytoplasmic cell differentiations. The peristome was always fimbriated and shed before any other apparent damage to the organism. Hence the most highly evolved differentiation of the cytoplasm is also the most sensitive, and the shedding effect seems to imply that the peristome is attached to the cell in a more vulnerable way than either the holdfast or the body cilia. The less specialized holdfast, which is only a temporary pseudopodial modification of the ectoplasm at the posterior pole [1] is the next to go; and finally the general ectoplasmic structure of the cell is affected.

When subjected to lethal concentrations of sea water stentors "die by inches". It is not the case that the cell as a whole gradually disintegrates, nor that sick cells are produced which are half dead throughout and later succumb completely. Instead there seems to be an advancing boundary between dead and living protoplasm such that up to the point at which about half of the cell is dead, the necrotic part can promptly be sloughed while the other part remains altogether intact and behaves like a cut stentor fragment, swimming about and soon regenerating missing parts. Furthermore, if a part of the surface of the cell showed disintegration of the cortical striping, the endoplasm enclosed also became necrotic and was cast off and never absorbed into the remaining unaffected portion of the cell. The advancing front of necrosis in both ectoplasm and endoplasm suggests that the intrinsic, vital organization of the cell is an all-or-none condition.

Comparable effects of Holtfreter's solution

Stentors responded to this mixture of salts about as they did to sea water. A brief exposure of 3 minutes invariably resulted in shedding of oral structures followed by regeneration of a new set in normal medium (24 cases). Several specific differences in reaction were noted, however. Immediately

upon introduction into Holtfreter's solution the stentors usually shed an outer layer of the cell which carried the imprint of the blue-green body stripes, though much of the pigmentation remained on the animals (as in Fig. 7). With longer exposure of over 5 minutes the whole anterior end of the cell disintegrated but the posterior pole was never visibly affected. Peristomial membranelles in the oral region were always the first to be sloughed; whereas in sea water the aboral end of the peristome went first and one could produce stentors without adoral bands but having the entire oral region intact. Conversely, in Holtfreter's, quickly stopping the treatment produced stentors which had cast off and ejected only that part of the peristome which extends into the gullet or had shed only the membranelles bordering the buccal cavity (Fig. 2). In either case, a whole new set of oral structures was regenerated.

Shedding of the oral primordium in lethal concentrations of sea water

The precursor of the peristome, the oral primordium, is also selectively affected by sea water. Twenty-seven dividing, reorganizing or regenerating stentors bearing oral anlagen were exposed to 25 per cent sea water for periods of time varying from 1 to 10 minutes. From the results (see Fig. 3) the following generalizations emerged. (*a*) Peristomes already formed were cast off first so that brief treatment caused shedding of old oral structures without affecting the primordia. (*b*) Well-formed anlagen were next to be sloughed, beginning (with few exceptions) at their anterior ends and progressing posteriorly. Primordium shedding was thus interpolated between two stages (*e* and *f*) in the sequence of salt effects outlined above. By stopping the treatment at a certain time, therefore, specimens could be prepared which lacked the original peristomes and a varying amount of the anterior parts of the primordia but retained the posterior portions intact. (*c*) The later the stage of development the more rapidly the primordia were affected. In dividing cells ready to begin separation, the advanced primordium of the posterior daughter cell was shed immediately following the casting off of the original peristome carried by the anterior daughter, but very early primordia in which the adoral cilia had not yet developed were not affected at all until the body ectoplasm—from which they emerge—itself began to disintegrate. (*d*) In all cases the treated specimens later regenerated the normal form. When the primordium was entirely sloughed a new one appeared; and if only the posterior portion remained it continued development, often producing correspondingly abbreviated oral structures (Fig. 3*a'*), as will be reported in detail in a later paper.

From these observations it can be concluded that a part of the oral anlage can go on developing as well by itself as when the whole primordium is present. Moreover, it is clear that cryptic effects of the salt dilutions do not occur if the part of the primordium not visibly affected demonstrates unimpaired capacity to continue differentiation, as was the case with but few exceptions. Especially significant seems to be the fact that the earlier the primordium the less rapidly it was affected by the treatment. Late anlagen resembled definitive peristomes in being rapidly sloughed, whereas early primordia were like the body ectoplasm in being disintegrated only after much longer exposure. Thus, from the time of its initial appearance to its complete differentiation into peristomial band and associated organelles, the primordium shows an increasing susceptibility to sea water. This change is consistent with the evidence that the oral anlage of *Stentor* begins as a derivative of the body ectoplasm [18] which later frees itself sufficiently to migrate to the anterior end as it matures in structure.

Peristome shedding and other reactions of Stentor *to lethal concentrations of certain salts, sugars, and other compounds*

Several pure salts and other compounds were tested with regard to their effect on *S. coeruleus*. Solutions were prepared as described above.

The major result was that every chemical tested except ethyl alcohol caused the stentors to shed the peristome within 20 minutes either in the solution or upon return to culture fluid. Only in the case of ammonium acetate and $NiSO_4$ did all the animals, which cast off the peristome, fail to recover from the treatment and regenerate a new set of feeding organelles when returned to normal medium. The compounds tested, concentrations used, and number of cases of recovery and regeneration after shedding of the peristome were as follows:

NaCl (1 per cent)—10 cases
$NaHCO_3$ (1.0–0.25 per cent)—18 cases
Na_2CO_3 (0.06–0.03 per cent)—7 cases
NaI (1.0–0.5 per cent)—32 cases
Sodium thiocyanate (1 per cent)—31 cases
KCl (1 per cent)—10 cases
KI (1 per cent)—12 cases
KNO_3 (1 per cent)—4 cases
$CaCl_2$ (1.0–0.5 per cent)—38 cases
$Ca(NO_3)_2$ (1 per cent)—12 cases
Calcium acetate (1 per cent)—13 cases
$MgCl_2$ (1 per cent)—8 cases
$MgSO_4$ (1 per cent)—4 cases
NH_4Cl (1 per cent)—10 cases
Ammonium acetate (1 per cent)—no recovery
LiCl (1 per cent)—8 cases
$NiSO_4$ (1 per cent)—no recovery
Strychnine sulfate (0.4 per cent)— 1 case
Sucrose (10 per cent)—20 cases
Dextrose (10 per cent)—10 cases
Albumin (0.12 per cent)—10 cases
Urea (1 per cent)—82 cases
Ethanol (16 per cent)—no peristome shedding

With the exception of the ethanol treatment, whenever a stentor was beginning division or reorganization and bore a late-stage primordium or precursor of the peristome, this was also selectively affected in every solution, but only after sloughing of the already formed peristome, just as in the case of sea water.

It seemed remarkable that such a variety of substances should produce the same reaction of peristome shedding. Yet there was some variation in the neatness with which this exfoliation occurred. In the sucrose or dextrose

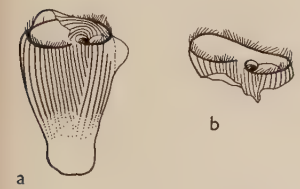
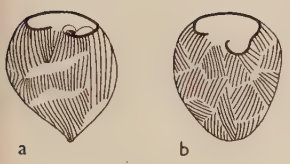


Fig. 4.



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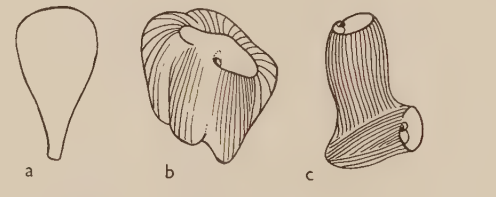

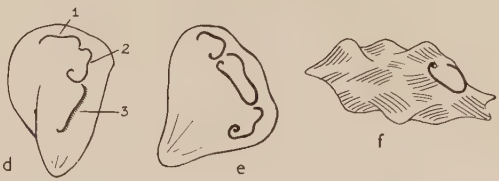

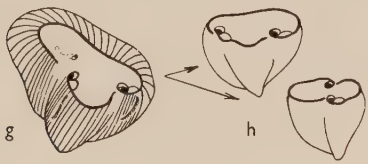

Fig. 6.

Fig. 4.—Reaction of *S. coeruleus* to ethyl alcohol. (*a*) Blisters formed and disintegration of ectoplasm beginning from posterior end; peristomial cilia still intact and actively beating. (*b*) Specimen with most of cell disintegrated but with oral structure still unaffected. This specimen recovered the normal form.

Fig. 5.—Reaction of *S. coeruleus* in long exposure to sublethal concentration of sea water. (*a*) After a few days lesions appear in the ectoplasmic striping. (*b*) Eventually this led to major disorganization of the stripe pattern; and astomatous regeneration of the oral structures is also shown.

Fig. 6. –Reactions of *S. coeruleus* to sublethal concentrations of LiCl. (*a*) Inhibition of regeneration—no primordium formed to regenerate missing oral structures. (*b*) Incomplete development of a regeneration primordium, the mouth being represented only by a small pit; broadening of the cell is indicated. (*c*) Daughter cells which failed to separate in division. (*d*) Specimen showing retention of 2 previously formed peristomes, while a third is now forming. (*e*) Three sets of incomplete oral structures accumulated in one broadened cell. (*f*) Stentor in which ectoplasmic striping has been broken into disoriented patches and cell shape is correspondingly abnormal. (*g*) Doublet cell with two sets of feeding organelles oriented around one adoral field. This specimen later reorganized doubly and divided doubly to produce two similar animals (*h*).

and in the urea solution the response was the nicest and was accompanied by the least other damage to the cell; for the adoral band was sloughed within 3 minutes, leaving sharp borders on the unaffected, adjacent adoral field and anterior body striping. Conversely, KCl, KNO_3, $MgCl_2$, egg albumin, and strychnine sulfate proved to be relatively unfavorable agents for this response as the majority of cells did not recover from the treatment.

The effect of ethanol on stentors was just the reverse of that of other compounds in that the body ectoplasm developed blisters and was progressively disintegrated from the posterior end of the cell, while the peristome was never shed and kept beating up until death of the organism (Fig. 4).

Shedding of pigment and of an outer layer of the cell.—In many of the solutions a portion of the blue-green pigment characteristic of *S. coeruleus* was sloughed, and when this occurred it was the immediate reaction of the animals to the alien medium. Pigment shedding and subsequent regeneration of full pigmentation after treatment with NaCl as described originally by Prowazek was confirmed. $NaHCO_3$, NH_4Cl, LiCl, strychnine sulfate, and albumin produced the most notable sloughing of pigment, but no shedding occurred in the Ca and Mg salts. Strychnine sulfate caused a violent reaction in which the pigment granules were shattered loose and largely cast out of the ectoplasm.

The pigment of *coeruleus* (see [8]) is present in rows of granules comprising the colored striping of the cell body. Since these granules, like those similarly located between the ciliary rows in other ciliates [7], showed a selective affinity for Janus green and because they gave a positive test for cytochrome oxidase, Weisz [19] concluded that they are probably mitochondria. It was striking in the case of strychnine sulfate and clear enough in many other solutions that the cast-off pigment remained in granular form. With several treatments, in fact, the sloughed pigment retained its original character as granular stripes (Figs. 7 and 8) which were lifted off the cell and remained intact in the medium. It was not possible to denude colored stentors of all pigmentation by repeated exposure to sloughing agents. And it should be

Fig. 7.—Pigment shedding of *S. coeruleus* in NH_4Cl, showing how the sloughed material retains the impression of the striping.
Fig. 8.—Cortical slough from *S. coeruleus* in albumin, showing separated pigment stripes.
Fig. 9.—Early disintegration of peristome (×) in LiCl while adoral field and lateral ectoplasm remains intact.
Fig. 10.—Membranellar band being shed in $NaHCO_3$, loosened first from the oral region.
Fig. 11.—*S. coeruleus* with peristome shed in sucrose solution.
Fig. 12.—Peristome shed by a *S. coeruleus* in sucrose solution, showing membranellar cilia and basement membrane.

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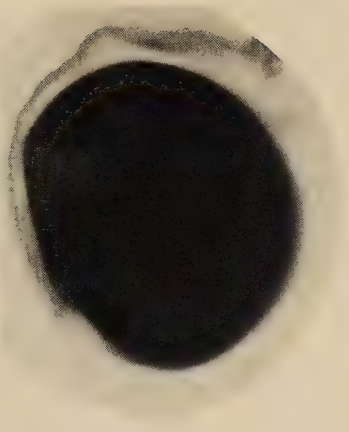

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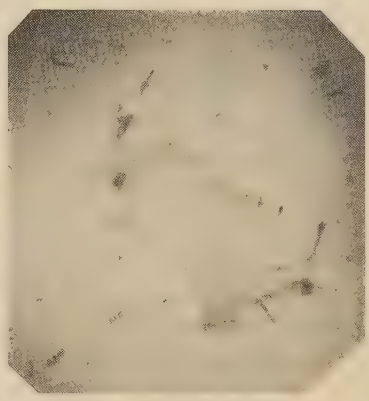

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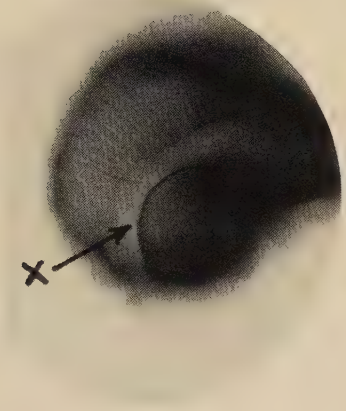

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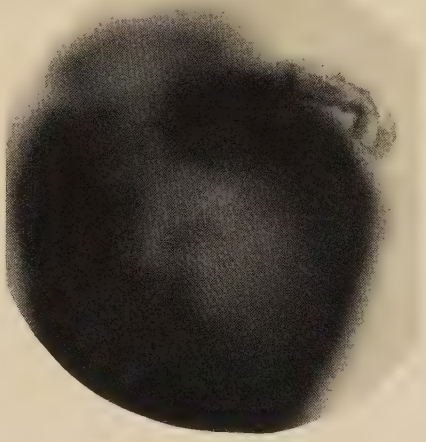

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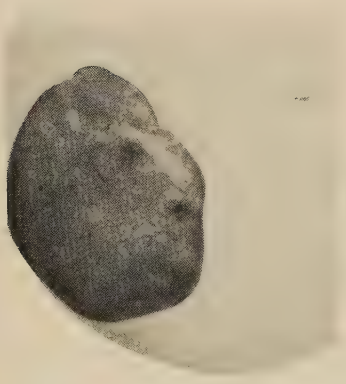

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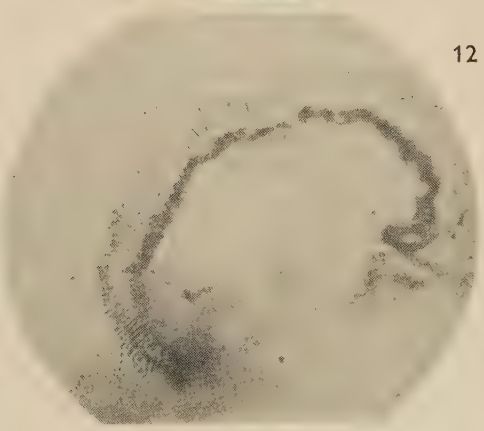

mentioned also that in the Na salts and mixtures the sloughed pigment appeared as a soft, diffuse halo—not granular.

In addition to pigment and pigment granules, an outer lamella was sometimes lifted from the cortex and carried off the end of the cell as a film by the beating of the body cilia. This was one of the effects of Janus green solutions described by Weisz [19]. The phenomenon recalls the shedding of the pellicle with its pigmented pink granules in *Blepharisma*, which Nadler [12] produced with salts of strychnine and morphine (although strychnine sulfate did not cause sloughing of the pellicle as a hull in the present studies with *S. coeruleus*). One is also reminded of the lifting of the fertilization membrane in eggs. Of the compounds tested, NH_4Cl, ammonium acetate, LiCl, and egg albumin frequently, but not invariably, caused the shedding of an outer layer of the cortex of the stentor cell (as in Fig. 8); and albumin likewise produced an ecdysis in the pigment-less *S. polymorphus*.

These reactions therefore suggest that the stentor cell may be useful in the experimental study of mitochondria, the lifting and the laminar nature of cell membranes, as well as the effect of the loss of outer coatings on permeability and immunological reaction; but a comprehensive exploration of shedding response was beyond the scope of this study.

Reversal of ciliary beating.—After pigment sloughing the next most immediate response of the stentors to certain chemicals was to swim backwards. Observation confirmed a major result of Merton's work [9, 10, 11], namely, that monovalent cations induce reversal of beating of the body cilia while bivalent cations do not. Partial ciliary anaesthesia by Na and K ions was also substantiated. Two observations may now be added to the extensive studies of Merton: (1) LiCl—which only disintegrated stentors for Merton—produced conspicuous backward swimming at 1 per cent concentration, and (2) 1 per cent ammonium acetate—which Merton did not test—caused the most prolonged and continuous reversal of any of the compounds used.

Reversible anaesthesia of myonemes.—The stentor cell is highly extensible and contracts vigorously through the action of myonemes lying beneath the rows of body cilia. An exposure of about 10 minutes to 1.0 or 0.5 per cent solutions of NaI and KI produced an anaesthesia of the myonemes so complete that cells could be cut in two without provoking any contraction whatever in either part. Yet this effect was reversible and full contractility returned after a day in normal medium.

Effects on the beating of adoral membranelles.—Some notable differences in the response of peristomial cilia to certain compounds were noted. In

NaI the membranelles remained continuously stopped, but in $CaCl_2$ they kept beating right up to the time of sloughing of the peristome.

In $NiSO_4$, peristomial and body cilia of *Stentor* are anaesthetized [15]; but though almost wholly quiet in themselves, the membranelles remained capable of vigorous reorientation. The points of these long cilia were directed backward and then forward alternately, corresponding to normal forward and backward swimming responses which were not carried out because of anaesthetization of ciliary beating. This apparent independence of ciliary beating and ciliary orientation is of interest in relation to the hypothesis of Fawcett and Porter [5], who have suggested that though ciliary beating is the result of reactions in the constituent fibers of the cilium itself, the striated rootlets attached to the basal body of the cilium are responsible for orientating the cilium and so determining its direction of beating. Since myonemes in *Stentor* are not anaesthetized by $NiSO_4$, the membranellar rootlets (also presumably striated) may remain unaffected to produce the reorientation of membranelles observed.

Sleigh [14] recently found that very low concentrations of $MgCl_2$ increase the frequency of beating but not the metachronal wave velocity in the membranelles of *S. polymorphus*. In the present study another interesting effect of this salt was observed in stronger solution. The membranelles stopped and then started beating about once per second. Peristomes watched in dark field therefore gave the appearance of continually flashing: obscure when active, bright and clearly visible by reflected light when stopped, the two phases continually alternating. Hence $MgCl_2$ might be useful in searching for a center of coordination of the membranelles.

Inhibition of the formation and development of the primordium by sublethal concentrations of sea water and of lithium chloride, etc.

Prolonged exposure to sublethal dilutions of sea water, LiCl, sucrose and Holtfreter's solution could prevent the formation of oral primordia or inhibit their development.

Survival of *S. coeruleus* was good in media to which sea water had been added to give a salinity up to 4 parts per thousand. The animals lived for about one week, which is normal for starving stentors, and they probably succumbed largely because of a partial ciliary anaesthesia which prevented their feeding normally. When normal animals were subjected to salinities of 3 to 4 p.p.t. there was a delayed shedding of the peristome, and after one day most specimens (21 cases) lacked feeding organelles. At these concentrations and down to $2\frac{1}{3}$ p.p.t. formation of a regeneration primordium

could be completely blocked, either in normal animals which had suffered delayed sloughing of the peristome in the dilute medium or in animals from which the adoral band had previously been shed in 25 per cent sea water. This inhibition of regeneration was reversible, for non-regenerating stentors completely recovered, regenerated normal feeding organelles, and could grow and divide when returned to normal culture medium (43 cases). Hence sea water not only causes the peristome and its anlage selectively to be disintegrated and sloughed but also can create a condition in which the oral primordium may not even be produced.

Complete but reversible blockage of anlagen formation was the extreme response. Lesser grades of effect were found in slightly more dilute solutions or in individuals presumably less sensitive to the stronger solutions of sea water, as follows:

(*a*) Primordium formation may merely be delayed. Thus in diluted sea water of salinity 1 p.p.t. primordia in all 7 cases did not appear until after 7 hours, instead of the normal time of 4½ hours.

(*b*) Primordia may form but develop incompletely, yielding peristomes without mouth-parts (3 cases—see Fig. 5*b*) or with very incomplete mouths consisting only of small pits (7 cases—similar to Fig. 6*b*).

(*c*) Primordia may be arrested in their development, remaining in place on the side of the cell and at least for a long time not continuing differentiation after the initial production of peristomial cilia.

(*d*) Aborted division may occur in which the daughter cells remain broadly connected, the fission line failing to complete its job of separation (2 cases).

(*e*) Finally, lesions in the ectoplasm of the cell body may occur which lead to distortions of the otherwise very regular stripe pattern of the cell (14 cases). In 5 extreme cases the effect led to marked disarrangements in which the striping was broken up into small patches oriented in all directions (Fig. 5). These specimens closely resembled minced stentors produced by cutting the ectoplasm into small pieces with a glass needle [16].

All the above effects were reversible in that the animals could recover, re-regenerate, and realign the body striping if returned to normal medium.

When stentors were caused to shed peristomes in 10 per cent sucrose and then returned to normal medium it was found that formation of regeneration primordia was delayed for 1 to 2 days (46 cases). This inhibition was due to some of the sucrose being carried over in the transfer; for if the animals were washed after the sucrose treatment to remove nearly all of the sugar they then regenerated in the normal time (95 cases). Hence sugar which in strong concentration causes shedding of the peristome, in sub-lethal

solution produces a marked delay in the formation of the anlage of the peristome.

One test was made using dilute (25 per cent) Holtfreter's solution. Delayed shedding of the peristome, astomatous development of the oral primordium, and stripe distortions were observed. Hence it was indicated that this mixture of salts, in sublethal concentrations of comparable strength, has long-time effects similar to those of sea water.

Of all the salts tested in lethal solutions, LiCl alone had a delayed effect. Stentors which had shed the peristome in 1 per cent LiCl and were returned to normal medium sometimes did not regenerate for 1 to 4 days, doubtless due to the carry-over of some salt on transfer of the organisms. A solution of 0.1 per cent LiCl was lethal to stentors which had previously been treated with 1 per cent LiCl, but not to those in which the peristome had been shed in sucrose solution, and therefore was close to the limit of tolerance. Below 0.005 per cent, LiCl produced no significant effects. At concentrations ranging from one of these values to the other, several effects on normal stentors or stentors required to regenerate by previous shedding of the peristome in sugar solutions were observed and are shown in Fig. 6.

(*a*) As with dilute sea water, LiCl could produce reversible inhibition of primordium formation, regeneration being blocked for 1 to 4 days but proceeding normally after the stentors were returned to normal culture fluid (Fig. 6*a*).

(*b*) The time of appearance of the primordium was sometimes merely delayed (7 ½ hours as compared with the normal period of 4 ½ hours).

(*c*) Development of the primordium was frequently arrested at the stage following formation of the peristomial cilia. Thus it would appear, as in the case of sea water, that at sublethal concentration the anlage may be blocked at just that stage when it becomes increasingly susceptible to the disintegrative effects of abnormal medium.

(*d*) Often the primordium appeared and developed a peristome which migrated into its definitive position at the anterior pole of the cell but no mouth-parts were formed, or only small pits, or ingestive structures clearly incomplete in their differentiation (Fig. 6*b*).

(*e*) Abortive fissions with daughter cells not separating but remaining broadly joined together were common (Fig. 6*c*).

(*f*) Distortions of body striping were sometimes found, but not as frequently as with sea water treatment (Fig. 6*f*).

(*g*) The distinctive feature of LiCl in its effect on the stentors was that it often produced a marked broadening of the cell or increase in cell diameter

without corresponding increase in length (Fig. 6*b*). Lateral body stripes were apparently increased in number, for there was no indication of swelling and the stripes were of normal density—not spread apart. In extreme instances this broadening led to the formation of doublet stentors (3 cases) capable of producing 2 primordia (Fig. 6*g*, *h*) which because of their form appeared not to be due to aborted fission in which the two sets of feeding organelles remain widely separated (cf. Fig. 6*c*). This effect was often accompanied by failure to dedifferentiate oral structures already formed so that in the course of successive reorganizations there was an accumulation of multiple peristomes (Fig. 6*d*, *e*).

Again all these effects were reversible, and normal animals could result after transfer to stock culture medium.

Fauré-Fremiet (with J. Ducornet) in 1949 [4] tested the effect of LiCl on *Leucophrys* (*Tetrahymena*) *patula* and found that a solution of about 0.1 per cent favors the production of the microstomatous form of this ciliate, apparently because of a special inhibition of multiplication of cilia in the paroral membranelle during its formation. It was suggested that this response is analogous to the vegetalizing effect of lithium salts on echinoderm development in which the proliferation of animal-pole cilia is suppressed. The results on stentor seem even more striking. LiCl can produce complete inhibition of the multiplication of cilia which produces the oral primordium, and may also result in astomatous differentiation of primordia, as well as the special effect of causing the cell-body to become abnormally broad; and all these effects are reversible.

Peristomial shedding in other species of Stentor

A sufficient number of tests were made to show that *coeruleus* is not unique in its reaction to abnormal media. The basic response of selective sloughing of the adoral band in diluted sea water and in sucrose solution, followed by recovery and oral regeneration, was also obtained with 2 other species.

A strain of colorless *S. polymorphus*, almost totally lacking the Chlorellae often found symbiotic in this species was used. Of 18 specimens treated 10 minutes with 10 per cent sucrose all reacted like *S. coeruleus* and shed all or part of the peristome or cast off the entire anterior end of the cell; 17 regenerated new oral structures, and only one died without recovery. The animals were, however, unusually sensitive to 25 per cent sea water; this mixture caused selective shedding of the peristome but of 24 specimens only 2 survived and regenerated while the rest died.

Similarly 14 specimens of a stock of *S. roeseli* shed peristomes in 10 per cent sucrose and all recovered and regenerated. In 50 per cent sea water, all 11 animals shed the adoral band and later regenerated.

SUMMARY

This study has shown that in the large ciliate *Stentor coeruleus* with its highly differentiated cortex the different components and derivatives of the ectoplasm react in specific and selective ways to various chemicals added to the medium. When lethal concentrations of the chemicals are used, these effects can be produced within a few minutes without further complications; and by watching the protozoa during the time prior to disintegration and returning them to normal medium at successive intervals, cells can be obtained which have suffered specific deletions from which they can recover.

Perhaps the most striking reaction produced was the selective shedding of the peristome and of its precursor, the differentiating oral primordium. How and why this occurs we cannot yet say, but the variety of chemicals which produced this effects seems to preclude certain lines of interpretation. That the effect was not ionic was shown by the fact that sugars also produced it and the reaction did not vary with the valency of ions in the salts tested. Similarly, the reaction apparently is unrelated to viscosity since Heilbrunn [6] showed that mono- and bivalent ions have opposite effects on the consistency of endoplasm and ectoplasm in *S. coeruleus*. The effect was probably not osmotic because very weak, non-hypertonic solutions of Na_2CO_3 and strychnine sulfate also produced peristome shedding, and in strong sugar solutions the peristome was sloughed long before the cell showed any sign of collapse due to osmosis. Hydrogen ion effects were ruled out because neutral substances were effective in producing this response. It would probably not be reasonable to regard sloughing of the adoral band as an autotomy since extreme mechanical injury does not produce it. The fact that ethyl alcohol alone of all the compounds tested had the opposite effect of causing initial destruction of the general body ectoplasm and never a shedding of the peristome may afford a clue toward the eventual explanation of this phenomenon.

The finding that sublethal concentrations of certain salt mixtures, sugar, and LiCl can cause complete or partial inhibition of the development of the oral primordium relates the effects of chemicals on *Stentor* to the vast problem of cell differentiation. Oral regeneration in stentors can also be inhibited by antisera (unpublished) as well as by anti-metabolites like acriflavin [20]; and incomplete, astomatous development of the oral primordium occurs in certain strains of *S. coeruleus* under normal conditions of culture, possibly because of genetic peculiarities [17]. It is likely that these lines of investigation will prove to be mutually illuminating.

A connection between morphogenesis and the reactions of *Stentor* to chemicals is further indicated by the fact that lithium produces special effects in this ciliate as it does in embryogeny. Suppression of both differentiation and dedifferentiation of feeding organelles of the "head" in *Stentor*, accompanied by a structural broadening of the cell which can tentatively be attributed to a stimulation of the multiplication of the lateral pigment and ciliary stripes, might be regarded as a result in this protozoan comparable to vegetalization in the sea urchin embryo.

Shedding reactions in respect to the pigment granules (mitochondria?) and outer layer of the cell membrane of *Stentor*, as well as specific effects on the behavior of the ciliary membranelles of the peristome were described. Besides posing interesting problems in themselves, these reactions provide a simple means of selective operation on cytoplasmic differentiations in this ciliate. It should be interesting to follow with electron microscopy the structural changes in the cortical differentiations of *Stentor* as affected by those chemicals which produced the most clear-cut reactions, and the fact that iodine salts were found to be perfect myoneme anaesthetics may make it possible to obtain suitable preparations for this purpose.

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CONTENT AND DISTRIBUTION OF ASCORBIC ACID IN SEA URCHIN EMBRYOS OF DIFFERENT DEVELOPMENTAL TRENDS

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Received March 27, 1957

The sea urchin egg contains within it the two types of antagonistic development—the vegetal, i.e. entomesodermal, type of development, with its center in the vegetal pole, and the animal, i.e. ectodermal, type, with its center in the animal pole [19]. These two types of development generally counterbalance each other, and the embryo develops into a normal pluteus larva. One or the other type of development may be favoured by microdissection technique or by treatment with chemicals [12]. Thus, treatment of the eggs with Li ions favours the vegetal development whereas treatment with SCN ions or *o*-iodosobenzoic acid (IBA) favours the animal development [1, 12, 16]. It has been the aim of many investigators to find as much data as possible about the biochemistry of these two antagonistic types of development [8, 16]. Any new observation may be valuable for the understanding of the biochemical background of differentiation. The present investigation serves as a contribution to this gathering of facts.

Some analyses of the ascorbic acid content in whole homogenates of normally developing sea urchin eggs were presented earlier [2]. The time course of the changes in ascorbic acid suggests that ascorbic acid is involved in the differentiation of entomesodermic tissues in the embryo. In the present investigation, two methods have been used to get further information about this question: a biochemical analysis giving the differences between the amounts in larval stages of different trend of development, i.e. containing different amounts of entomesoderm, and a histochemical method showing the detailed localization in the larvae. The former method is quantitative, whereas the latter is merely qualitative.

MATERIAL

The chemical part of this investigation was carried out on fresh or lyophilized sea urchin material from *Paracentrotus lividus* at the Laboratoire Arago, Banyuls-sur-Mer, France. The histochemical studies were made on the rather transparent

eggs and larvae of the shallow form of *Psammechinus miliaris*, at Kristineberg's Zoological Station, Fiskebäckskil, Sweden. The samples were collected from artificially fertilized eggs, cultivated in glass trays on a rocking board according to Lindahl [16]. For obtaining vegetalized and animalized larvae, the eggs were treated as follows. The vegetalization was brought about by a 3-hour treatment of the eggs, starting at the 8–16 cell stage, with a 10–15 per cent solution of LiCl, isotonic to sea water, i.e. 0.59 *M* for 3.7 per cent salinity at Banyuls and 0.40 *M* for about 2.5 per cent salinity at Kristineberg. After the Li treatment, the larvae were repeatedly washed in sea water and finally transferred to another solution of sea water for further cultivation [5]. For animalization, the eggs were subjected to a permanent treatment starting immediately after fertilization with $4–8 \times 10^{-4}$ *M* IBA in sea water [1]. Some batches of animalized eggs were obtained by treatment with *M*/80 thiomalic acid [15].

RESULTS

Analyses for ascorbic acid and the separations of the free and the bound form were carried out according to the methods of Roe and Kuether and of Sumerwell and Sealock [18, 21]. The micromethod of Glick, Alpert, and Stecklein was applied to the sea urchin material [7]. The amount of ascorbic acid in the extracts was referred to the Kjeldahl nitrogen value of the lyophilized powder. When analyzing fresh material, reference was taken to a certain number of eggs. When describing the variations in the ascorbic acid content during development, development is here divided into two phases, the first involving the development to about 20 hours after fertilization, and the second the late development to about the 48 hours stage.

The first phase is characterized by alternating small decreases and increases in the ascorbic acid content. The values oscillate about 20 γ per γ *N* in normal and in abnormal stages. Larvae at different developmental stages show small characteristic differences. As a consequence of the slow developmental rate of animalized and vegetalized larvae, the small wavelike variations are retarded in the abnormal larvae. In animalized larvae the amount is lower than that of normal ones immediately after fertilization, increases at hatching, and decreases again. In the vegetalized larvae the amount is generally lower than in the normal larvae.

The bound ascorbic acid, 1–2 γ per γ *N*, which is present in the unfertilized egg slowly disappears during the cleavage stages.

The second phase of development, i.e. after the 20 hour stage is characterized by a series of rapid and sharp increases and decreases in the ascorbic acid content in the normal larva [2]. Similar but even more pronounced increases and decreases occur in the vegetalized larvae. Here, the ascorbic acid content is generally higher and the peak values always exceed those of the normal larvae. Ascorbic acid seems to be accumulated at that time when the most vegetal part of the embryo wall is being prepared for the

following exogastrulation. During exogastrulation the extra supply is lost. One wave of variation in the diagram, consisting of one peak and one valley, thus seems to correspond to the formation of one exogastrulated vesicle.

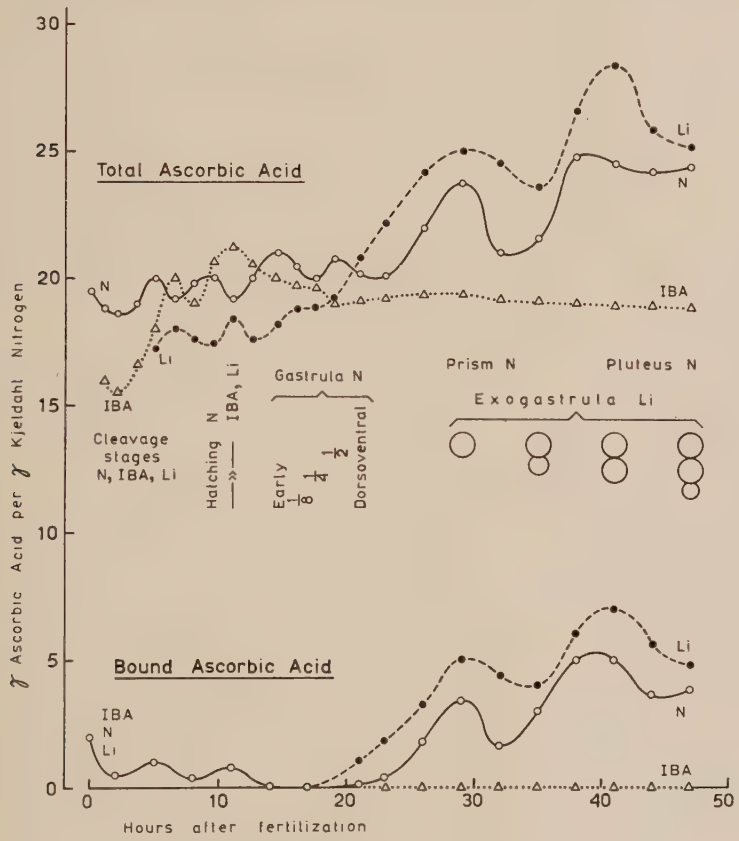


Fig. 1.—Content of ascorbic acid, total amount and bound fraction, during the development of normal, vegetalized and animalized sea urchin larvae (*Paracentrotus lividus*).

In the animalized larvae the ascorbic acid content remains on the initial low level or decreases somewhat. The same results are obtained irrespective of the animalizing agent: IBA or thiomalic acid.

The bound ascorbic acid, ascorbigen, reappears during the second phase of development in normal and vegetal, but not in animal larvae. Most of the changes in the total ascorbic acid content during this period are due to increases and decreases in the ascorbigen. Consequently more ascorbigen is synthesized in vegetal than in normal larvae and none appears in animal

larvae, where no increase occurs in the total ascorbic acid. Note, however, that the content of free ascorbic acid is also subjected to changes during this period, the highest amount being present in vegetal and the lowest in animal larvae.

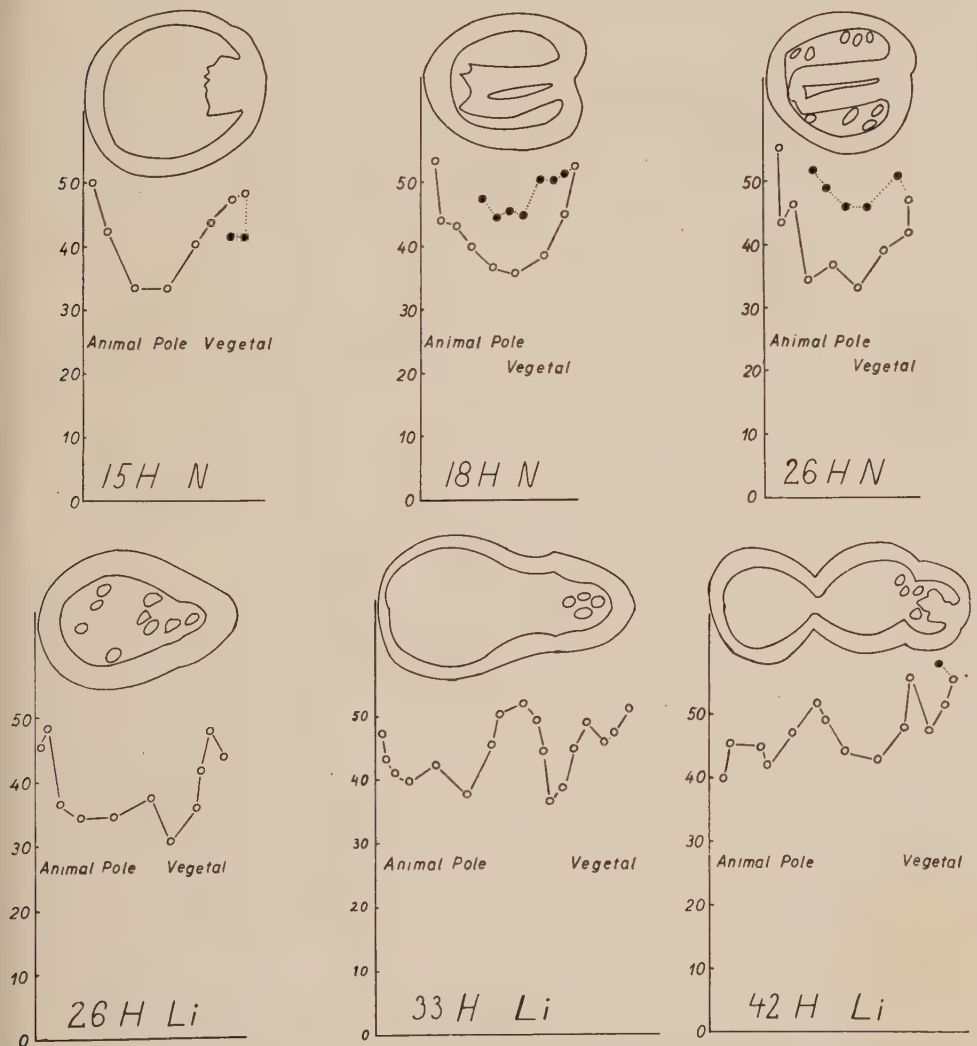
The analyses described above were mainly performed with the 2,4-dinitrophenyl-hydrazine method for the total ascorbic acid. To obtain some information about the proportion of reduced and oxidized ascorbic acid, some differentiated analyses were also made on fresh sea urchin material according to the 2,6-dichlorphenol–indophenol method of Glick, Alpert, and Stecklein for reduced and the above mentioned method for total ascorbic acid [7]. These analyses revealed, however, no measurable difference between the amounts of reduced and total ascorbic acid, i.e. almost all the ascorbic acid must be present in the reduced state.

The ascorbic acid in ascorbigen was previously supposed to be protein-bound [2]. Histological studies in plants indicate a linkage between ascorbic acid and ribonucleic acid [13]. Sea urchin material, from which the free ascorbic acid was extracted, was treated for two hours with ribonuclease, and this treatment released about 50 per cent of the bound ascorbic acid. This experiment speaks in favour of a relation between ascorbic and ribonucleic acids in the sea urchin as well.

It may be observed that higher amounts of ascorbic acid were found by the present analyses, carried out on fresh or newly lyophilized material, than by the previously reported analyses [2]. The differences may be due to the destruction of the free ascorbic acid in the previous material, analyzed about 18 months after lyophilization. The amount of ascorbigen is, however, the same, indicating a higher stability during storage of the bound than of the free form.

The distribution of ascorbic acid in the sea urchin larvae was studied by a silver impregnation method, mainly according to the prescriptions and suggestions given by Eränkö [6]. The eggs and larvae were treated for 20 minutes with a mixture of 10 ml 20 per cent $AgNO_3$, 2 ml 96 per cent alcohol and 1 ml glacial acetic acid. Afterwards they were repeatedly washed in distilled water, slightly acidified by acetic acid. Precipitated AgCl and non-reduced Ag^+ ions were removed by 30 minutes of treatment with 5 per cent sodium thiosulphate, the solute being changed some two or three times. After a new period of intense washing with distilled water, the eggs and larvae were passed through the dehydrating alcohol series to bensol, embedded in paraffin wax and sectioned in 10 micron thick sections. The sections, mounted on slides, and counterstained with paracarmin or eosin, were studied with an immersion objective 60× and an ocular 15×. The ascorbic acid sites were now blackened by silver precipitations with a granular size of about ½–1 micron. A small network of 20 × 20 squares was inserted below the ocular. The number of silver granules was

counted in as many squares as possible, through the whole thickness of the section, by slowly rotating the micrometer wheel. Each value was plotted on a drawing of the section. From this drawing fully plotted with figures, a diagram was made of the zonal distribution of the ascorbic acid along the animal-vegetal axis of the egg.



Figs. 2–7.—Gradient curves showing the ascorbic acid distribution along the animal-vegetal axis from sections of embryos of different age. *N* denotes normal larvae, and *Li* larvae vegetalized by Li ions; age in hours (*H*); filled and open circles indicate the number of silver granules counted within a square of the network; open circles and whole lines refer to results from surface cells of the larvae; filled circles and dashed lines refer to results from invaginated cells. (From *Psammechinus miliaris*).

The ascorbic acid sites are evenly distributed during all cleavage and blastula stages of the developing sea urchin egg. In the very earliest cleavage stages, when the blastomeres still are large, the ascorbic acid is localized around the nucleus, but is absent from its immediate neighbourhood. Possible changes connected to fertilization have not yet been studied.

At the beginning of gastrulation the ascorbic acid sites are concentrated at the poles, especially at the vegetal pole of the embryo. In later stages, high concentrations also occur in those parts of the invaginated entoderm which are adjacent to the blastopore. Later, all of the ectoderm except for its most animal part loses much of its ascorbic acid and the sites are more densely distributed in the entoderm, especially in the blastopore area and in the most invaginated tips of the intestine.

In the late vegetalized blastula, i.e. 20–25 hours after fertilization, the ascorbic acid sites are concentrated at the most vegetal part of the embryo. When the first exogastrulated entoderm vesicle is formed, the ectoderm vesicle loses much of its ascorbic acid, except in the most animal area. Centers with accumulation of sites are formed in the entoderm vesicle. In the animal part of this vesicle there is an annulum of ascorbic acid sites and in the most vegetal tip there is another accumulation where the formation of a second exogastrulated intestine vesicle is prepared. When this is formed, the sites are distributed within it in the same way as just described for the preceding vesicle in an animal annulum and a vegetal tip. Several zones of ascorbic acid accumulation are thus arranged in the intestine of the vegetalized exogastrula. It is most likely that a similar distribution occurs also in the normal pluteus larva. (The analyses of the ascorbic acid content in whole larva homogenates speak in favour of this.) The conditions are, however, much easier to study in the vegetalized larvae with their elongated animal-vegetal axes than in the normals. Both in the normal and in the vegetal larvae the ascorbic acid sites become more concentrated in the entoderm as the larvae ages. In the animalized larvae, where development goes no further than the blastula stage, the sites of ascorbic acid are also evenly distributed in the late stages.

DISCUSSION

In the present study the ascorbic acid content has been investigated in several stages of normal, vegetal, and animal sea urchin larvae with chemical and histochemical methods. The small differences between normal and abnormal larvae during the first 20 hours of development are mainly due to a general retardation during the abnormal development. The decreases and

increases of the cyclic changes are synchronous with similar phases in the content of free amino acids, SH-containing substances (mainly glutathione), and carotenoids [3, 4, 14]. It seems as if the ascorbic acid content during the first 20 hours of development reflects a general rythmic metabolic pattern.

The results from the later stages of development indicate a gradient in the ascorbic acid content directed towards a vegetal trend of development. The older the larvae become, the richer is the accumulation of ascorbic acid in their vegetal parts. The high ascorbic acid content of the vegetal and the low content of the animal larvae is in good agreement with the finding that glucoascorbic acid, an ascorbic acid analogue, causes animalizing tendencies [9].

It was shown in other material that high amounts of ascorbic acid are accumulated when periods of rapid growth occur in certain types of tissues, and these are correlated with high amounts in desoxyribonucleic acid [17, 20]. The same pattern also seems to occur in vegetal cell types in the sea urchin development. In animal larvae with a low content of ascorbic acid, the development ceases at the blastula stage, while the normal and vegetal larvae continue to develop. The growth of entomesodermal tissues is even more pronounced in the vegetal larvae during exogastrulation than in the normal larvae during differentiation and late development of the pluteus larva. Ascorbic acid is accumulated during the preparatory phase and broken down during the stretching phase of an exogastrulation wave. A parallel phenomenon may be supposed to occur during invagination in the normal larva. Accumulation of ascorbic acid precedes the invagination. During the first rapid phase of invagination, part of it is broken down. A new accumulation occurs during the morphological standstill, followed by a new loss during the continued invagination [10].

The presence of bound ascorbic acid, ascorbigen, in normal larvae was dealt with in the previous paper [2]. The chemical data indicate a localization of the bound form to structures containing ribonucleic acid but the present histochemical method does not permit any conclusion about the localization of ascorbic acid in certain cell structures. From the histochemical and chemical studies it seems clear, however, that the accumulation of ascorbic acid is limited mainly to entomesodermic, i.e. vegetative differentiations. It may be observed that ascorbigen reappears at about the same time as the red pigment, echinochrome, begins to be synthetized in the sea urchin larva. This pigment is also present in higher amounts in vegetalized than in animalized larvae [4, 11].

Most, if not all, of the ascorbic acid is present in the reduced state in the sea urchin. The ascorbic acid eventually plays an important role for the

maintenance of a reduced state in the sea urchin larvae, especially in the cells of the most vegetal parts of the embryo. No parallelism occurs, however, between ascorbic acid and other substances in the reduced state, e.g. glutathione and protein bound SH, during the late phase of development [3]. It must thus be supposed that it is the presence of reduced ascorbic acid *per se*, and not the presence of a reduced state which is of importance to vegetal development.

SUMMARY

1. Ascorbic acid was determined by chemical and histochemical methods in several stages of normal sea urchin larvae, as well as in larvae with enlarged or reduced entomesoderm.

2. After small cyclical changes during cleavage and blastula stages, ascorbic acid is accumulated during the phase of entomesodermal development and restricted to entomesodermal cells.

3. These results are discussed in relation to the accumulation of ascorbic acid which is observed during the development of certain cell structures.

This work has been carried out with supports from the Royal Academy of Sciences and from the Swedish Natural Research Council. The author wishes to express his most cordial thanks to Professor John Runnström and to Dr. Tryggve Gustafson for the great interest they have shown in this work. He also owes a great debt of gratitude to the authorities at the Biological Stations in Banyuls and Kristineberg.

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COUNTING ACTIVELY METABOLIZING TISSUE CULTURED CELLS[1]

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Received April 8, 1957

Previous metabolic experiments on tissue cultured cell suspensions [10] indicated that values obtained on different cultures varied more than replicate determinations on the same suspension. In addition, metabolic quotients decreased if the culture medium was not renewed. These observations led us to suspect that tissue cultures contain a variable number of dead cells. As a consequence an attempt was made to estimate live-dead cells by eosin staining procedures [3, 9] which have been used in spermatozoa studies.

It was difficult to differentially stain tissue cultured cells by these methods because the cells stained three ways. Supposedly, live cells did not stain and dead ones stained red, but some cells stained pink when dried slides were made. When stained in suspension, all of the cells progressively took up the dye. The concentration of Eosin B used was 2 mg/ml cell suspension.

In a recent report [17] Hoskins used Eosin Y to differentially stain the ascitic form of Krebs 2 carcinoma. He used the stain in a final concentration of 0.5 mg/ml cell suspension and found live cells to be pink and dead ones red. One of his conclusions was that *in vitro* methods can give very inaccurate estimates of viable cell count. From these reports and others it appeared that the concentration of eosin used was generally too great for staining cells in suspension and consequently made difficult an accurate differentiation. By reducing the eosin concentration to 0.20 mg/ml it was found that some cells took on a red color almost instantly and other cells did not stain. Furthermore, the number of non-stained cells did not appear to change.

Kaltenbach and Kaltenbach [8] found that the glycolytic acitvity of ascites cells was due only to the live cells present. The cells were killed by placing them in a hypotonic solution. Differential live-dead staining was done by the method of Schrek [12] which incorporates eosin in a final concentration of about 0.95 mg/ml.

[1] Supported by Ladies Aux. Vet. Foreign Wars, Nebraska Chapter.

The primary objective of this study was to see if eosin or one of its derivatives could be used to differentially distinguish actively respiring and glycolyzing tissue cultured cells. Some of the possible limitations of the method were also investigated.

EXPERIMENTAL

Earle's Strain L and Gey's Strain HeLa cells were used for study. They were grown in T-60 [5] flasks in a medium [11] containing 0.25 g lactalbumin and 0.25 g yeast enzymatic hydrolysates (Nutritional Biochemical Corp.) per liter Earle solution [4] plus 100 ml human serum. Medium was renewed three times weekly. All cultures proliferated very rapidly and had been subcultured almost every week for more than a year.

Cell suspensions were prepared in the following manner. The cells were carefully washed with Earle solution while still attached to the floor of T-60 flasks. They were then covered with about 10 ml of Earle solution and dislodged with a brush made of stainless steel and nylon bristles. Clusters of cells were separated further by filling and discharging them ten times through a 3.5 inch 18 gauge needle. The syringe plunger was completely inserted into the barrel only on the tenth mixing so that a minimum of cells would be crushed.

Initial trial and error experiments indicated that 20 mg per cent Erythrosin B (actually 19.1 mg per cent) gave a very satisfactory contrast of stained to non-stained cells when a green filter was used on a microscope lamp. This stain was found to be better than Eosin Y, Eosin B or Phloxine B. A differential count was made by adding 0.05 ml of 0.4 per cent Erythrosin B in water to one ml of cell suspension, a drop of which was introduced by capillary attraction into a hemacytometer counting chamber. The cells were given three minutes to settle to the ruled surface and then both stained and non-stained cells were counted in a 1.0 mm square. Since these squares have a volume of 0.1 mm^3 one has only to multiply the count by 10,000 to determine the number of cells per cm^3. Correction is made for the 0.5 ml of Erythrosin added initially to the ml cell suspension.

Experiments were arranged to see if the number of non-stained cells was correlated with metabolic activity of the cell suspension. In order to do this some of the cells had to be killed. This was done by exposing one half of a cell suspension in an open petri dish to ultraviolet light for 30 minutes or by heating to 53°C in a water bath for 15 minutes. Neither procedure completely killed all cells. Subsequent experiments revealed that cells killed either way showed the same staining characteristics. Varying concentrations of live and dead cells were mixed so that each suspension contained about the same number total cells. Respiration was determined by the direct

method on a Barcroft-Warburg respirometer [13] at 37.5°C in a phosphate buffered physiological solution [11] for an hour. Anaerobic lactic acid formation was determined as previously described [10] in Earle solution by the chemical method of Barker and Summerson [1]. Stained and non-stained cell counts were made at the beginning and end of a determination. Results were based on the mean of the initial and final count. While determining

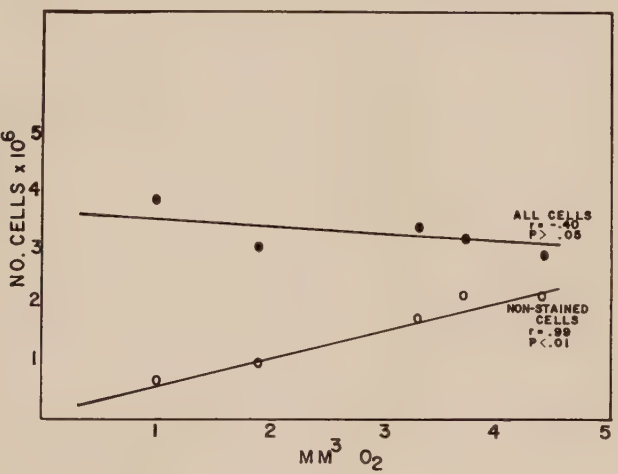

Fig. 1.—Respiration of Strain L Cells not staining with 20 mg per cent Erythrosin B.

lactic acid formation, the number of stained cells increased slightly when exposed to an atmosphere of 5 per cent CO_2 in N_2 but the change was about the same as that observed for determination of respiration in air. Respiration was found to be positively correlated ($r = 0.99$, $P < 0.01$) with the number of non-stained cells (Fig. 1). In contrast to this finding there appeared to be little relationship between total cell count (stained plus non-stained) and respiration ($r = -0.40$, $P > 0.10$). Eight determinations were made for anaerobic glycolysis and it was found that lactic acid formation was also correlated with number of non-stained cells ($r = 0.88$, $P < 0.01$). As with respiration there was little relationship between total cell count and glycolysis ($r = 0.32$, $P > 0.10$). These results are evidence that Strain L cells not stained by 20 mg per cent Erythrosin B actively metabolize whereas those stained do not.

The accuracy of a hemacytometer count depends primarily on the number of cells counted [2]. To have less than 5 per cent error more than 1000 cells usually have to be counted. For this reason it was desired to find out how long Strain L cells in Earle solution could be exposed to Erythrosin at room temperature before there was a significant change in the number of stained

cells. A cell suspension with Erythrosin was prepared as described. Stained cells in this suspension were counted at intervals up to 100 minutes. As can be seen from Fig. 2 (Controls) there was not a significant change in the number of stained cells for more than half an hour.

The above experiment was repeated using trypsin instead of a nylon brush to dislodge the cells since trypsin is used to prepare cell suspensions

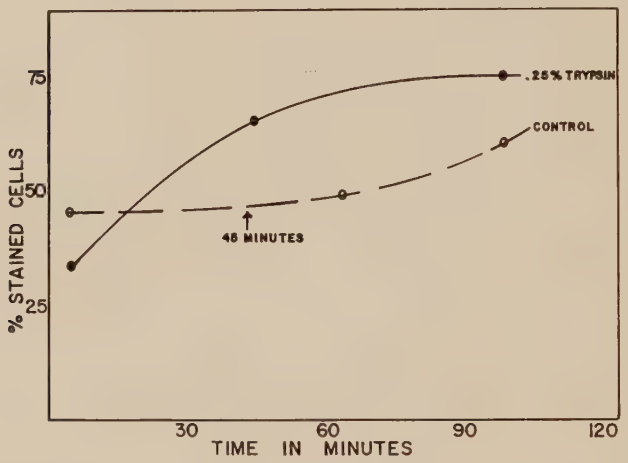

Fig. 2.—Increase in stained cells treated with 20 mg per cent Erythrosin B in Earle solution.

[14]. A 0.25 per cent solution of Difco (1:250) Trypsin in Earle solution was used to free the cells which were then collected by centrifugation (140 G) and washed in Earle solution. The number of cells taking up Erythrosin increased with time (Fig. 1). However, trypsin treated cells did not stain the same as cells in suspensions prepared with a nylon brush. Instead of taking up the stain almost instantly they gradually increased in color. It appeared that trypsin changed the permeability of the cells and they slowly took up the stain.

Tissue cultured cells sometimes have to be collected by centrifugation to obtain an adequate concentration of cells for metabolic studies. For this reason the effect of centrifugation on the percentage of non-stained cells was tested. Arbitrary aliquots from the same suspension were centrifuged at different speeds for 10 minutes, then resuspended and the percentage of non-stained cells determined. It was found that centrifugal speeds up to 1800 rpm (690 G) did not decrease the percentage of non-stained cells (Table I). At 2800 rpm (1650 G) there were fewer non-stained cells although the difference was not significant ($P > 0.70$) as compared to the controls which were not centrifuged.

TABLE I

Effect of centrifugation and pH on the percentage of non-stained cells.

	Total no. cells $\times 10^4$	No. det.	% non-stained cells
Cent. (Strain L)			
Control	120	27	59.2
140 G	89	18	59.1
470	87	9	59.2
690	77	9	59.7
1,650	74	18	54.1
pH (Strain L)			
6.7–7.0	90	2	58.5
7.1–7.4	99	2	70.4
7.5–7.8	66	2	73.5
7.9–8.2	111	2	58.4
pH (HeLa)			
6.1–6.5	23	3	43.5
6.6–7.0	69	2	52.2
7.1–7.4	64	2	79.5
7.9–8.3	35	2	37.6

Two experiments were performed to note the effect of pH on tissue cultured cells (Table I). In one experiment, a suspension of Strain L cells in Earle solution was divided into 8 aliquots and the pH adjusted on a Beckman Model G pH meter with 2.28 per cent $NaH_2PO_4 \cdot H_2O$ or 1.75 per cent Na_2HPO_4. After exposure to these solutions for three hours the cells were collected by centrifugation and resuspended in Earle solution (pH 7.4). A similar experiment was done using HeLa cells which were exposed to various pH for one hour. As might be expected, there were more non-stained cells in pH range 7.1–7.8. The number of stained cells increased greatly on either side of this range.

Cell suspensions made in balanced saline solutions were found to contain 25–50 per cent stained cells which seemed high for an actively growing tissue culture. We thought that the sudden change of the cell environment from culture medium to balanced saline might partially account for these observations. To check this hypothesis cell suspensions were prepared in four different solutions and differential counts made on each (Table II). There were about 62 per cent non-stained cells in suspensions prepared in balanced saline solutions. One saline solution was buffered with bicarbonate

TABLE II

The effect of suspending solution on the percentage of non-stained cells.

Suspending solution	Buffer	No. cultures	% non-stained cells
Balanced saline	HCO_3	4	61.8
Balanced saline	PO_4	6	62.0
Culture medium	HCO_3	3	88.7
Culture medium	PO_4	3	86.3

(Earle solution) and one was buffered with phosphate [11]. The type buffer used did not appear to affect differential staining because the number of non-stained cells was about the same in either solution. In order to differentially stain cells in tissue culture medium containing serum and hydrolysates 0.2 ml of 0.4 per cent Erythrosin B had to be used for each ml of cell suspension. Suspensions prepared in culture medium containd about 87 per cent non-stained cells whether the media were buffered primarily with phosphate or bicarbonate. Again the buffer did not appear to influence differential staining in physiological pH ranges. About 25 per cent more non-stained cells were obtained when cell suspensions were prepared in tissue culture media than when prepared in balanced saline solutions.

DISCUSSION

Error involved in counting animal cells can be estimated by the formula of Berkson, Magath and Hurn [2],

$$V = \sqrt{\left(\frac{100}{N_b}\right)^2 + \left(\frac{4.6}{N_c}\right)^2 + \left(\frac{4.7}{N_p}\right)^2},$$

where V is the coefficient of variation in per cent, N_b the total number of cells counted, N_c the number of counting chambers used, and N_p the number of diluting pipettes. In a typical experiment to determine the respiration of Strain L cells three T-60 flasks containing 10–15 million cells each were suspended in about 25 ml of balanced saline. A total of 1292 non-stained cells were counted in 9 squares (0.1 mm^3) on three counting chambers which were filled with 3 pipettes. Substituting these figures in the formula the error involved in counting was about 4.7 per cent. Actually this figure may be high because diluting pipettes were not used. Pipettes were used only for transferring the cells to hemacytometers.

Most staining experiments were carried out in balanced salt solutions. If soluble proteins are present an excessive amount of Erythrosin has to be used. Protein solutions tested were serum, Difco trypsin, and yeast and lactalbumin hydrolysates. Probably proteins bind the dye [6], thus decreasing the amount available for cell staining.

A variety of other cell types were used to study their staining characteristics. Erythrosin B as used in these experiments was found to differentially stain: frog spermatozoa, exfoliative cells in human and frog urine, leucocytes which had been slightly heated, protozoan and metazoan parasites in frog urine, both dry and wet brewer's yeast, fungi (Roqueford cheese), trypsin prepared cell suspensions from dog kidney, spleen or testis, HeLa cells and some bacteria. Eosinophils or erythrocytes obtained from fresh blood apparently were not stained with the concentration of Erythrosin used.

SUMMARY

In balanced salt solution 20 mg per cent of Erythrosin B differentially stained Strain L tissue culture cells. Cells that were not stained respired and produced lactic acid. Stained ones did not metabolize. Those cells that were not stained almost instantly with Erythrosin resisted being stained for more than a half hour when the suspension of cells was prepared with a nylon brush. Centrifugal speeds up to 2800 rpm did not appear to be detrimental to the cells. The number of metabolizing cells was greatly decreased outside the range pH 7.1–7.7. Suspensions of Strain L cells in balanced saline solutions contain more stained cells than suspensions in tissue culture medium containing serum and lactalbumin and yeast hydrolysates.

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SUSTAINED CELL CULTURE

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Received April 10, 1957

Tissue culture is of great value in many biological and biochemical studies because it provides an opportunity to observe growing homogeneous cellular systems under defined conditions. The technique would be even more widely applicable, however, if the cells could be propagated under uniform conditions wherein alimentation would be continuous without diminution in concentration of dietary essentials and where injurious or nonessential cellular metabolic products would be speedily removed. The usual flask or tube cultures are not only limited in size, they are also subject to wide fluctuation of the cellular milieu; the cells are alternately feasted and fasted. The various expedients which are employed to diminish these artefacts, such as frequent renewal of the medium or explantation of the culture, fall short of the desired goal, which is to enable the experimenter to alter one variable at a time. In addition, continuous cultures would appear to offer advantages over the usual discontinuous cultures as stock repositories of viable cultures and for experiments of long uninterrupted duration such as may be of value in some virus studies.

This paper describes a continuous regulated cytogenerator, which, in some degree, approximates intact animal conditions, and in which cells can be propagated for long periods of time. Permeable membranes are employed for introducing nutrients and for removing cellular waste products, while controlled pulsatile gas pressures maintain gas exchange and keep the cells dispersed. The volume of the culture is kept constant, but specimens may be removed as desired without disturbing the composition of the medium or sensibly affecting the growth rate. Cultures of Earle's strain L, clone 929, fibroblast cells [25], and of a preparation of Ehrlich ascites cells [15], have been propagated in this device in logarithmic phase for as long as thirty days. There is no reason why growth could not continue for a considerably longer time if larger volumes of media were prepared at the outset. The limits of cell concentration are still under study. We have succeeded in culturing ascites cells directly from the peritoneal cavity of the mouse where

others have failed [2], or have achieved only limited success [27], but the maximum concentration of ascites cells obtained so far, falls short of that observed in the peritoneal cavity of the mouse.

Our arrangement departs in principle from those hitherto described for the uninterrupted propagation of bacteria or fungi [19, 21], in which a constant population is maintained by the balanced addition and removal of medium and the continuous elimination or harvesting of the crop. Our device, however, maintains growth constancy without loss of cell mass, and so facilitates incorporation and turnover studies on the total cell population, particularly with reference to compounds consumed in minimal amounts. Cells can be propagated at a fixed rate in sufficient mass to permit the use of N^{15} tracers.

Cells in static tissue culture systems have a tendency to adhere to each other and to other receptive solid surfaces. Indeed, this property might be one of the controlling factors in tissue formation and growth *in vivo*. These cells propagate in sheets because nutritional access is limited by the rate of diffusion through the cellular layer. The introduction of innocuous cellophane strips or glass helices [10, 11] is effective in increasing the attachment surface without proportionate increase in medium volume. The most efficient situation, however, is that in which the cells are in free suspension so that the entire cell surface is in contact with the medium. The application of our device is limited to those cells which can be grown in free suspension, but the number of cells which cannot be adapted to free suspension conditions appears to be small and determinable only by trial. It must not be overlooked, however, that some properties of cells arise from syncitial or colonial contact. It can be opined, though, that the free suspension artifice, a departure from the natural metazoan state, facilitates energy exchange. That some cells have been grown hitherto on solid surfaces only and not in free suspension is perhaps an indication that other limiting cultural or mechanical desiderata have not been found or provided.

It can be inferred heuristically that optimal physical conditions are to be found *in vivo*, as in ascitic fluid, for example, where concentration gradients are minimal. (It cannot be inferred, however, that ascites fluid is superior to dietary mixtures which have been or can be prepared, for there is good evidence that only those cells which have attained a high degree of autonomy, and hence have an exalted capacity to survive in a relatively poor medium, are capable of conversion to an ascites type.) Vigorous mechanical agitation of the culture flask with a fixed volume of medium under continuous oxygenation has been applied successfully by Earle [9] in whose hands the medium

underwent considerable change in composition in a short time, however, necessitating frequent removal of the spent medium and equally frequent replacement with fresh medium. All discontinuities have been avoided in the arrangement described below, the mechanical equivalent of a heart-lung-kidney preparation.

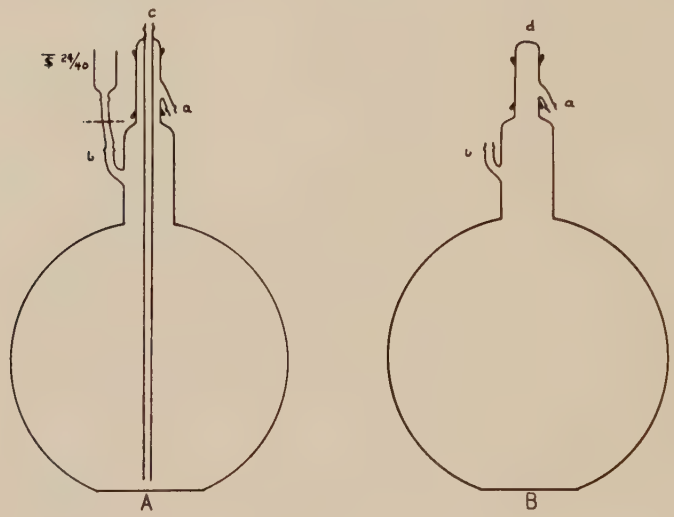


Fig. 1.—Medium reservoirs. *a*, sterile air vents; *b*, 24/40 *S* joint adapter for selas candle; *c*, siphon arrangement. Flask *B* is the spend medium receiver.

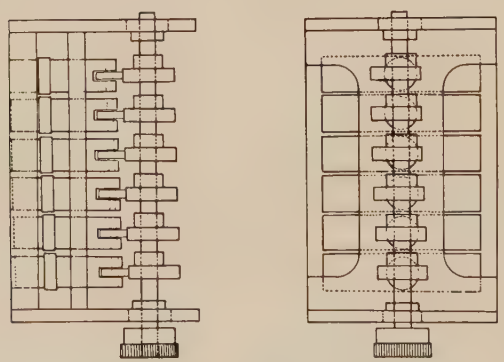


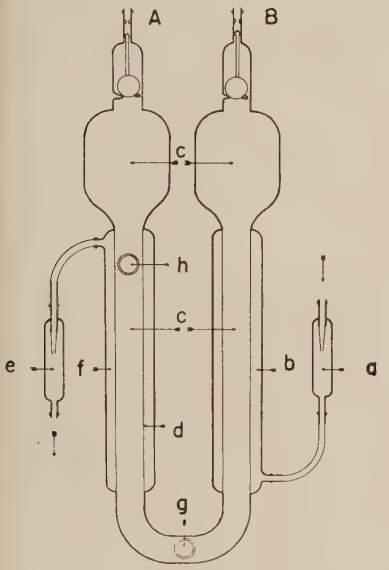
Fig. 2.—Perfusion pump with six compression cams.

MATERIALS AND METHODS

The apparatus consists of three main units, *I*, a reservoir for the nutrient medium, a peristaltic pump, drop counters, and a reservoir for spent medium, *II*, an all glass culture flask with fritted glass walls, *III*, a mixed gas source with Venturi valves, sterilizing filters, a pulsatile gas pump, and a sterile exhaust train.

The reservoir for the sterile medium (Fig. 1 *A*) is kept at 4°C; the medium is delivered to the culture flask through sulfur-free latex tubing by means of a positive action

perfusion pump driven by a geared Bodine Motor. (Motor 2T60-18, manufactured by Gerald K. Heller Co., Baltimore 23, Maryland.) The arrangement permits regulation of pumping speeds from 3 ml per hour to 300 ml per hour. The pump is made up of six finger-type compression bars (Fig. 2) producing a forward thrust of the medium in the lumen of the tube. Unidirectional motion is assured by the sequential arrangement with at least two bars simultaneously compressing the tubing. (Manufactured by



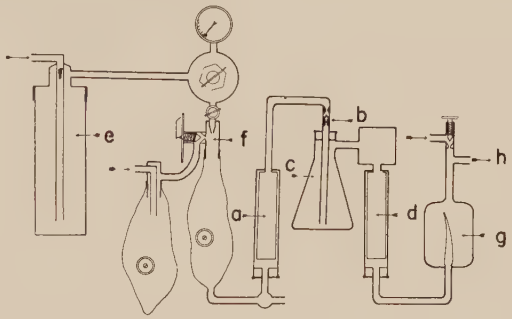


Fig. 3 *(left).*—Glass cytogeneration flask. *a*, *e*, drop counters; *b*, *f*, outer jackets; *d*, fritted glass candles; *g*, *h*, "no hole" serum stoppers.

Fig. 4 *(right).*—Schematic arrangement of gas supply. *e*, gas filter; *f*, Venturi valve; *a*, Berkefeld candle; *b*, Ringling Ros valve; *c*, water saturator; *d*, Berkefeld filter; *g*, glutter valve; *h*, house vacuum.

Frederick Sellmer, 80 East 236th Street, New York 70, New York.) The medium is propelled through a drop counter (Fig. 3*a*) to the outer jacket of the culture flask (Fig. 3*b*). The solution passes through the medium porosity fritted walls of the culture flask and mixes with the contents of the cell compartment proper (Fig. 3*c*). The spent medium is filtered through a second fritted glass wall (Fig. 3*d*), to a second drop counter (Fig. 3*e*) and then through the perfusion pump in a reverse direction to the inflow, to regulate the removal of medium in conformity with the inflow. The medium is continuously exchanged while the cells are agitated in a solution of constant volume. The pump delivers the spent medium to an exhaust flask (Fig. 1*B*) which is similar to the fresh medium reservoir flask. Provisions for sampling the perfusion medium and for the addition of tracers or other compounds has been made (Fig. 3*h*).

The glass culture flask is essentially a continuous U tube, with medium porosity fritted glass filter cylinders constituting the two upright arms. (Manufactured by E. Machlett and Son, 220 East 23rd Street, New York 10, New York.) Both ends of the U-tube (Fig. 3*A* and *B*) are connected to sterile gas supply lines through glass float valves. The fritted filter cylinders are jacketed by glass sleeves in a condenser arrangement (Fig. 3*b* and *f*) with inlets and outlets. Access to the outer jacket of the candle is provided by means of a "no hole" serum stopper (Fig. 3*g*). This part of the apparatus is housed in an incubator cabinet.

TABLE I

Basal medium for suspension cultures[a]
(in m*M*/liter).

(L)-Amino acids		Co-factors		Inorganic salts	
Arginine	0.10	Ascorbic acid	0.3000	NaCl	119.0
Cystine	0.05	*p*-Aminobenzoic	0.0010	KCl	5.36
Histidine	0.05	*d*-Biotin	0.0010	$MgSO_4$	0.81
Isoleucine	0.20	Choline	0.0100	NaH_2PO_4	1.46
Leucine	0.20	Folic acid	0.0010	$NaHCO_3$	14.50
Lysine	0.20	*m*-Inositol	0.0010	$CaCl_2$	1.76
Methionine	0.05	Nicotinamide	0.0010	$ZnSO_4$	0.003
Phenylalanine	0.10	Ca Pantothenate	0.0010	$Fe(NO_3)_3$	0.002
Threonine	0.20	Pyridoxine	0.0010	$CoCl_2$	0.0002
Tryptophane	0.02	Riboflavin	0.0001	$MnCl_2$	0.0005
Tyrosine	0.10	Thiamine	0.0010	$CuCl_2$	0.0004
Valine	0.20	Vitamin B_{12}	0.0001		
Glutamine	1.00				

[a] Glucose 11.1 m*M*/liter. Dialyzed horse serum, see text.

The medium porosity fritted glass walls through which the medium passes are impermeable to cells. Ideally, however, these walls should be semi-permeable, except for the fact that unresolved components, either contained in, or associated with, high molecular weight serum proteins, are as yet still indispensable for cell culture. The fritted glass candles around which the cell chamber is constructed, were evolved from experiences with graded collodion membranes, which, incidentally, are very difficult to sterilize without impairment. It was at first anticipated that denser candles would be necessary, but medium porosity candles proved to be adequate for retention of all the cells tested so far. Only minimal resistance to flow is encountered when medium porosity is used. The fritted glass walls serve a dual purpose; not only do they admit nutrients to the cells, they also take out spent medium and cellular waste products, which can be harvested or sampled at intervals for analysis. Some cellular products or materials adventitiously introduced with the original culture are so inhibitory as to preclude successful culture unless they are removed.

Our limited initial objective, that of obtaining maximum cell proliferation rates over a long period of time, necessitated modification of media commonly employed in discontinuous culture [5, 6, 7, 12, 16, 22, 30]. The composition of the medium used is shown in Table I. Amino acid-free horse serum, prepared by passage through Dowex 1×10, chloride cycle, and Dowex 50×10, sodium cycle, and dialysis against the inorganic components of the medium was added to the listed low molecular weight components in amounts of 5 to 20 per cent. It was found that the generation time of cells cultured in such mixtures is influenced by the concentration of serum proteins added, the more protein, the shorter the generation time, all other factors equal. Protein nitrogen determinations indicate a utilization of the protein. This mixture is

certainly over-generous in some ingredients and just barely sufficient in others, but it served us well in the initial experiments and provides a standard for detailed study of nutritional requirements. It is anticipated that continued experimentation will lead to further definition and simplification of the medium.

One of the important limiting factors so far encountered is that of glucose supply; glucose is a limiting factor even in static cultures and cannot be supplied in high

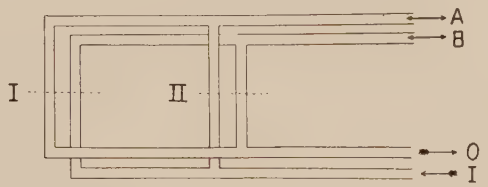


Fig. 5.—Gas alternater.

enough initial concentrations without producing deleterious osmotic effects. In an effort to measure glycogen production by strain 721 and strain L cells, Westfall *et al.* [28] observed that the glucose concentration of their medium decreased to zero in a very few hours. Westfall *et al.* [29] found also that static suspended HeLa cultures consumed as much as 2.49 g of glucose in 24 days to yield but 8 g wet weight of cells. Such a consumption of glucose is equivalent to that of a 70 kg man using 901 g of glucose per day. Gemmill *et al.* [13] found a Walker rat sarcoma 319 culture to take up glucose at the rate of 0.85 mg of glucose for 10.2 mg of tissue per day. This uptake of glucose is comparable to that of a 70 kg man using 5.810 g of glucose per day! The respiratory quotient of 1.0 observed in a variety of tissue cultures [23] and the demonstration of the presence of all of the tricarboxylic acid cycle enzymes in HeLa cultures [1] illustrates the predominance of glucose as the energy source for tissue culture biosynthesis. The inflections of the growth curves for strain L cells and for ascites cells in Figs. 4 and 5 are the results of inadequate glucose supply in the face of increasing cell populations, even with increased perfusion rates. Measures to compensate for this deficiency with heavy cultures by adding glucose separately from auxiliary reservoirs are under study.

The inordinate glucose consumption of tissue cultures does not strictly reflect the energy outlay, however, because much of the glucose consumed is degraded only to lactic acid. Glycolysis is disproportionately high in tissue cultures, and apparently all tissue cultures, whether of normal or malignant cell origin, bear some resemblance to intact tumors in the animal host and to slice preparations of tumors where the Pasteur effect has been found to be minimal. It is improbable that growing cells rely on glycolytically generated energy alone, even though the ATP yielded in the degradation of glucose to lactic acid reaches considerable magnitudes by virtue of the sheer mass of glucose consumed.

Tissue cultures are very sensitive to oxygen tension, and variation in oxygen tension is soon reflected in the cell generation time and in survival. Cellular outgrowth ceases altogether at oxygen concentrations of less than 0.5 ml per liter of medium (atmospheric oxygen tension of about 2 per cent) [17]. It will be interesting to observe whether or not the enhanced glycolytic capacity of cells which can be

cultured *in vitro*, could enable the cells to survive without replication at oxygen tensions below those necessary for replication. Excessive oxygen is deleterious also; concentrations of over 23 ml per liter, as may be found under 95 per cent oxygen atmospheric pressure, are cytotoxic [3, 18, 20].

It is necessary to provide carbon dioxide as well as oxygen, both for maintenance of pH and for incorporation. Tissue cultures appear able to survive, although not to propagate, under rather wide variations of pH 7.4, but the lag phase can be eliminated completely by adjustment to pH 7.0. Not only is the CO_2/Na HCO_3 buffer system a very poor one; it is also unstable in the direction of increasing alkalinity unless the correct gas tension of CO_2 is maintained in the manner provided for in the cytogenerator. Attempts to replace the CO_2/Na HCO_3 system by other, more efficient, buffer systems are unsuccessful [26], or permit only limited survival [8]. It appears that cells have a nutritional need for CO_2 above and beyond buffering action. The CO_2 might be needed to form oxaloacetate from pyruvate, or even perhaps to form 2.2-hydroxymethyl-glutyryl-CoA by condensing with 2-hydroxyvaleryl-CoA [24] for example.

The gas mixture is applied directly to the cell suspension in alternating pulses on both limbs of the U-tube vessel for the purpose of mixing the contents without frothing. Agitation can be regulated by varying the rate and the magnitude of the displacement. The gas source can be either commercially available compressed mixtures of O_2, CO_2, and N_2, or laboratory compressed air coupled to a CO_2 tank through a Venturi valve. Compressed air and tank CO_2 are mixed in a Venturi-regulated proportioning balloon (manufactured by Respiration Aids Co., 255 East 148th Street, New York, New York) which permits adjustment of the CO_2 content between 0.04 and 20 per cent.

The desired gas mixture is saturated with water vapor. (The solution contains 10 per cent ethylene-diamine-tetraacetate and 0.05 per cent phenol-sulfonphthalein. The solution is adjusted to pH 7 with NaOH) after passing through a sterile Berkefeld candle *N* protected by a "Ringling Ros" glass valve (manufactured by Ringler Rados Surgical Corp., 3958 Broadway, New York, New York) (Fig. 4*b*). The sterile water-saturated gases now enter the alternating device shown in Fig. 5. Two motor-driven cams, geared to alternately compress two tubes simultaneously while they open two others (Fig. 5*A* and *B*), permit the gas mixture to flow to side *A* when *I* is closed, and to release gas at side *B*; when *II* is closed, the gas flows to side *A*. The gears are so arranged (Fig. 6) as to provide a complete cyclic pulsatile change of gas every 5 seconds, a rate which has been found to suffice for adequate gas exchange and cell movement. The gases are vented through a sterile exhaust train composed of a second Berkefeld filter and one-way flutter valve connected to the house vacuum through a by-pass needle valve. Appropriate collection traps can be inserted in this line when analysis of spent gas is desired. The magnitude of fluid traverse in the culture flask is determined by the pressure differential in the two limbs of the culture flask.

The problem of initial sterilization and the maintenance of aseptic conditions over long periods of time is a formidable one which exerts considerable influence on the design and size of reservoir flasks, physical accessibility to the pumps, etc. The self-contained and connected units are steam sterilized. The flexible rubber tubing, with sterile candles, water-vapor saturator and solution (Fig. 4*a*, *c*, *d*), cytogenerator, medium reservoir flasks and medium exhaust flask are connected as illustrated,

and the whole unit is autoclaved at 15 lbs pressure for 30 minutes, preferably repeated on two successive days to avoid spore formation.

The medium is sterilized afterwards by pressure filtration through a #03 selas candle contained in a glass sleeve, provided with a male 24/40 T joint to fit the medium reservoir. After sterile filtration, the rubber tubing is clamped off, and the filter removed. Antibiotics are usually unnecessary if adequate sterile techniques

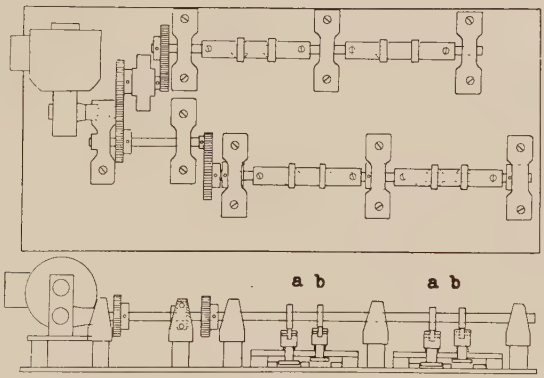


Fig. 6.—Gas pulsation pump for four separate systems. *a*, *b*, compression cams.

are observed. If mouse ascites cells are to be transferred directly from the peritoneal cavity, however, it is wise to include 100 units of penicillin G-Na and 100 mg dihydrostreptomycin/ml.

RESULTS

The cells so far grown in this apparatus are pure cultures of strain L, clone 929, fibroblasts; HeLa cells [26]; strain KB, and Chang [4] liver cells; and an impure Ehrlich ascites strain carried in C_{57} mice in our laboratory.

The growth of three continuous homeostatic cultures of strain L cells in 5 per cent horse serum is shown in Fig. 7. Experiment B-95 was started with an inoculum of 625 mg wet weight of cells. The wet weight of cells was computed from the count. In eighteen days, the wet weight increased to 8.0 g wet weight of cells, 4.3×10^6 cells per ml, with a total cell population of 2.0×10^9 cells. The generation time was 117 hours. In a duplicate experiment, C-3, an inoculum of 924 mg wet weight of cells, 9.6×10^5 per ml, increased in seventeen days to 8.2 g wet weight of cells, 4.5×10^6 cells per ml, with a total cell population of 2.1×10^9 cells. The generation time was 129 hours. A short term experiment, C-7, started with an inoculum of 440 mg, 244,000 cells per ml, increased in 14 days to 3.0 g cells, 1.7×10^6 cells per ml, a total cell population of 7.5×10^8. The generation time was 121 hours. However, when 10 per cent horse serum was employed in another experiment, the generation time was reduced to 41 hours.

Fig. 8 depicts the growth rate of Ehrlich ascites cells under our *in vitro* conditions. These cultures were started with 7-day peritoneal fluid from C57 mice in which the strain has been carried by serial passage. An inoculum of 696 mg, 6.2×10^5 cells per ml, gave rise to 5.0 g, 2.3×10^6 cells per ml, and total cell population of 1.2×10^9 cells, in fourteen days. The cells were

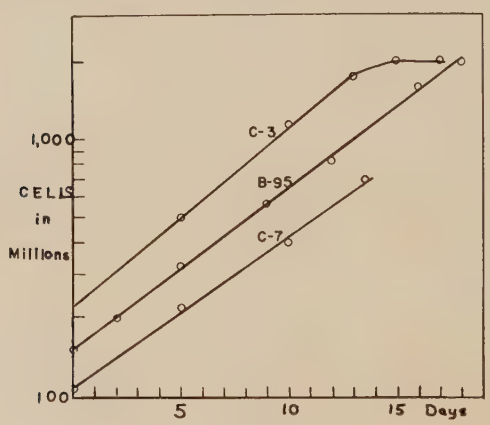


Fig. 7.

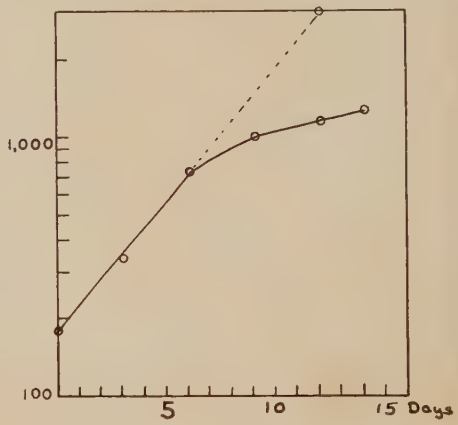


Fig. 8.

Fig. 7.—Growth of Strain L, clone 929, cells in cytogenerator. Abscissa, time in days; ordinate, log of cell number.
Fig. 8.—Growth of Ehrlich ascites cells in cytogenerator. Abscissa, time in days; ordinate, log of cell number.

always spherical in form; microscopic search did not disclose any degenerating elongated spindle forms such as have been described for failing *in vitro* preparations. That the biological properties of these cells were apparently unaltered by their *in vitro* sojourn was demonstrated by re-inoculation into C57 mice which were killed in seven to eight days as before.

The generation times of the cells cultured in suspended state compare favorably with those reported and found by us on static cultures, using 10 per cent horse serum, Strain L cells in static culture in a "complete" medium (20 per cent WEUF, 40 per cent BSS), exhibit a generation time of 25 hours over brief culture periods. If, however, these cells are cultures in a more defined medium, such as Eagle's [5], which is more appropriate for metabolic study, the generation time is prolonged to 48 hours. Suspended cultures in the cytogenerator with the modified Eagle medium, afforded a mean generation time of 120 hours for *prolonged periods.* The various factors influencing generation time are under further investigation toward a goal of 25 hours or less, as is obtained with the crude medium. As previously indicated, horse serum may constitute one of these factors.

SUMMARY

Gas exchange, introduction of nutrients, and removal of waste products can be regulated in a new device for continuous sustained culture of cells in suspension.

Strain L, clone 929, fibroblasts; HeLa cells; and strains KB, and Chang, liver cells were successfully propagated in this device.

Ehrlich ascites cells have been propagated *in vitro* for the time.

We wish to express our gratitude to Dr. W. R. Earle for his generosity in supplying us with an initial culture of fibroblasts, and for his friendly advice.

This work was supported by a grant from the Damon Runyon Memorial Fund.

This report was submitted to the Faculty of Pure Science, Columbia University, by Kenneth S. McCarty in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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HETEROCHROMATIC REGIONS AND NUCLEOLUS ORGANIZERS IN CHROMOSOMES OF THE MOUSE, *MUS MUSCULUS*

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Received April 12, 1957

Although structural features of mammalian chromosomes such as the primary and secondary constrictions have been well studied in the past several years [11, 15, 18, 21, 22], the heterochromatic regions of mammalian chromosomes have received relatively little attention. It seemed therefore desirable to attempt a correlation of the mammalian chromocenter with specific regions of the chromosomes. The present paper presents some observations which indicate that the chromocenters of mouse nuclei may be related to the heterochromatic regions of individual chromosomes.

MATERIALS AND METHODS

A survey of various cell types of the mouse has revealed that the spermatogonium is the cell best suited for a study of this kind. Testes were obtained from new-born and full-grown mice of the CBA, AKR and A/Heston strains of the species, *Mus musculus*. Critical observations of morphological details were made on squash preparations prepared by the following two procedures:

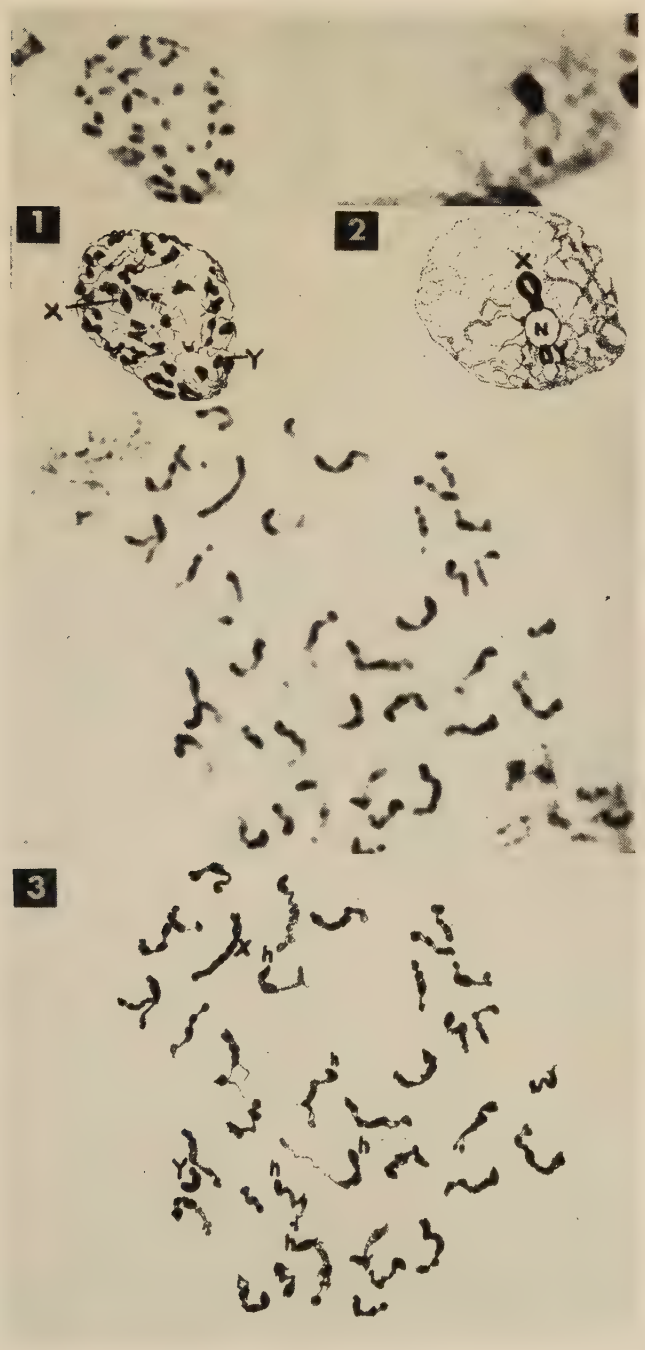
(1) For the study of mitotic chromosomes, several seminiferous tubules freshly removed from the sacrificed animal were minced in a drop of distilled water placed on a glass slide. After sufficient mincing, excess pieces of tubules were removed and a drop of glacial acetic acid was added to the cell suspension thus obtained. A silicone-

All the photomicrographs have been taken by Leitz Panphoto 15 × 90. *h*- - - -, heterochromatic region of autosome; × - - -, the X chromosome; *Y* - - - -, the Y chromosome; *N* - - - -, nucleolus (or sex vesicle.)

Fig. 1.—One type of a spermatogonial interphase nucleus from the squash preparation stained with Feulgen alone. 40 chromocenters are observed. The two chromocenters which are especially heteropycnotic are believed to represent the X and the Y chromosomes.

Fig. 2.—A second type of a spermatogonial interphase nucleus from the same preparation. The nucleolus is shown as the Feulgen negative body in association with the totally heteropycnotic X and Y chromosomes.

Fig. 3.—A spermatogonial late prophase stained with Feulgen. The X and the Y chromosomes are distinguished by their totally heteropycnotic nature. Each of the 38 autosomes has a heterochromatic region close to the proximal end. One small autosome is not included in this photomicrograph.


1
X
Y
2
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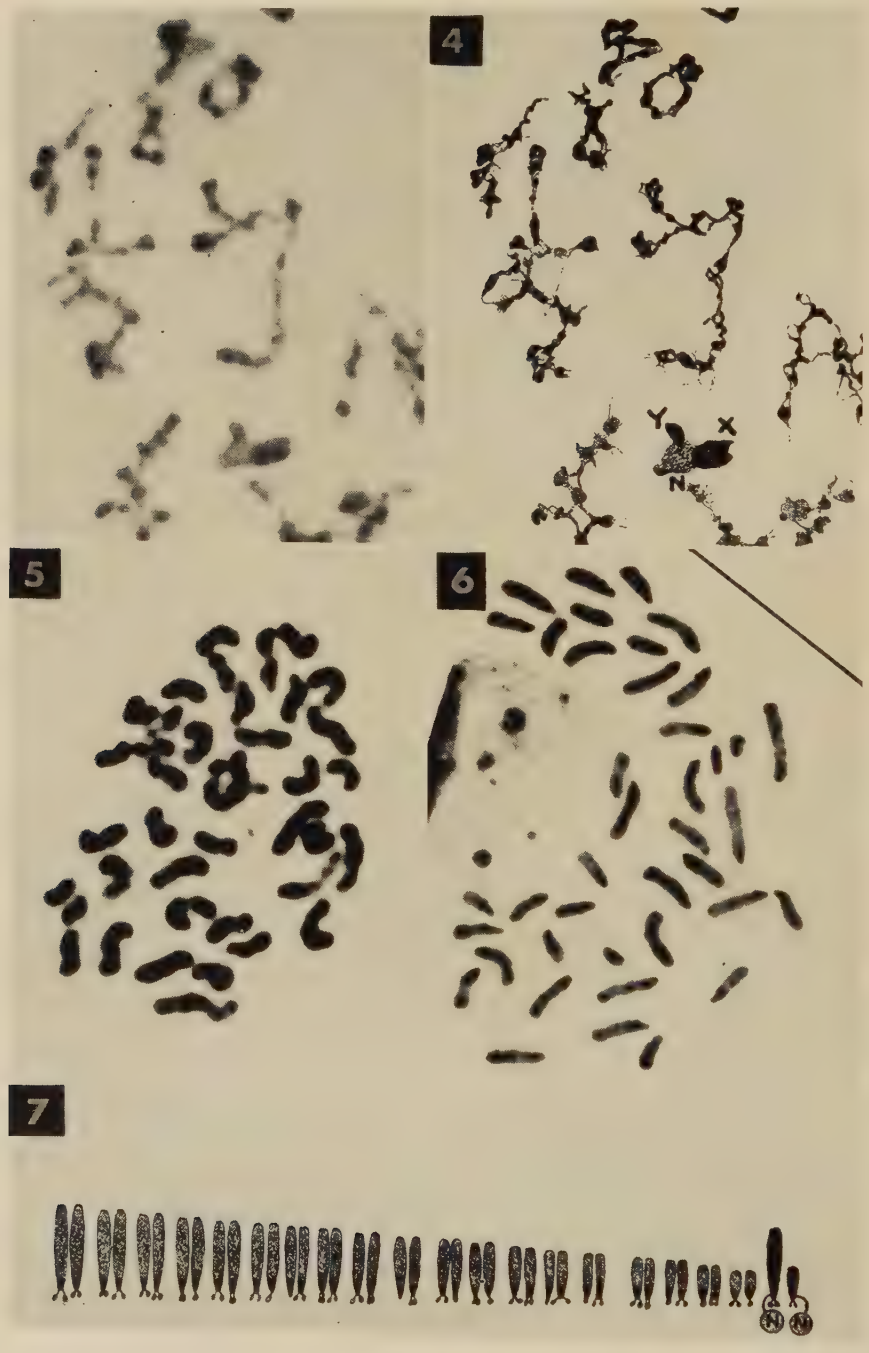
3


4
Y
X
N
5
6
7

coated coverslip was placed on the fixed cell suspension and the cells were squashed firmly between the coverslip and the glass slide. Excess fixative expelled by this process was removed by means of filter paper. The coverslip and glass slide were separated from each other by immersing them in 5 per cent acetic acid solution for 10 minutes. Most of the cellular material remained fixed to the slide surface. The squash preparations were stained by the Feulgen method.

(2) For the study of interphase nuclei, several seminiferous tubules were minced in a drop of cold saline instead of distilled water, thus avoiding the hypotonic effect. In order to preserve the nucleolus, Carnoy fixative was used instead of acetic acid. The procedures following fixation were the same as described above. A counter stain of 1 per cent light green solution was occasionally employed for delineating the nucleolus.

OBSERVATIONS

The heterochromatic regions of the spermatogonial chromosomes were most clearly recognized in the A/Heston strain of mice. Thus, the following descriptions are based largely on the findings using this particular strain:

Spermatogonial interphase (*Figs. 1 and 2*).—The karyosomes or chromocenters of interphase nuclei of the mouse are generally variable in number and size. In the case of the spermatogonial nucleus, however, the chromocenters showed remarkable consistency in number and size. This was particularly true in the A/Heston strain of mice.

Two different types of spermatogonial nuclei have been observed. One type is small, has no nucleolus and contains numerous chromocenters which, in well-squashed nuclei, where it is possible to count the exact number of chromocenters, are always 40 in number. Among these 40 heterochromatic bodies, two may be distinguished by their higher degree of heteropycnosis. By their behavior in subsequent mitotic phases they may be identified as the X and the Y chromosomes. The other 38 chromocenters may be identified as the heterochromatic regions of the 38 autosomes in subsequent mitotic phases (Fig. 1).

The second type of nucleus is large and vesicular, possessing a single

Fig. 4.—A late diplotene figure stained with Feulgen and light green. The totally heteropycnotic X and Y are attached terminally to the Feulgen negative sex vesicle which is actually the nucleolus.
Fig. 5.—A spermatogonial prometaphase stained with Feulgen. The 38 autosomes are in process of condensation.
Fig. 6.—A spermatogonial metaphase stained with Feulgen. All the 40 chromosomes are equally condensed. A heterochromatic region adjacent to the kinetochore may still be recognized on some of the autosomes.
Fig. 7.—A schematic drawing of alignment with the heterochromatic regions painted black. All of the 40 mouse chromosomes have the kinetochore at the region extremely close to the proximal end. The 38 autosomes have the small heterochromatic region adjacent to the kinetochore. The X and the Y chromosomes are entirely heterochromatic having the nucleolus organizer at the region adjacent to the kinetochore.

large nucleolus. The nucleolus is always found in association with two deeply stained heteropycnotic bodies, one larger than the other. In well-squashed nuclei, these two bodies may be seen as chromosome-like two-stranded bodies, each attached terminally to the nucleolus. The marked difference in size of these two bodies suggests that they are the X and the Y chromosomes in totally heteropycnotic state (Fig. 2).

Spermatogonial prophase (*Fig. 3*).—Late prophase figures permit a critical analysis of the heterochromatic regions of all chromosomes. The heteromorphic XY pair is marked by its total heteropycnosis. The large one probably represents the X and the smaller one the Y as has been observed previously. In sharp contrast to the totally heteropycnotic X and Y, the 38 autosomes are still in a rather stretched state, staining unevenly and showing a varying number of constrictions. Each of the 38 autosomes, however, shows a pycnotic area in the region close to the kinetochore at the proximal end of the chromosome (Fig. 3).

The effort to observe a definite association between the totally heteropycnotic X and Y and the nucleolus at early prophase has met with failure. This is probably due to the fact that most of the mitotic prophase figures are derived from the type of nuclei described in Fig. 1 which do not have a nucleolus. However, such an association may be observed during the meiotic prophase where the X and the Y of the mouse are known to be totally heteropycnotic [20]. A definite association of the heteropycnotic X and Y to the nucleolus is clearly evidenced at the late diplotene stage of meiosis (Fig. 4).

Spermatogonial metaphase (*Figs. 5 and 6*).—During prometaphase, condensation of the autosomes takes place (Fig. 5), with the result that the 38 autosomes become equally condensed with the X and the Y chromosomes. Thus, it is almost impossible to distinguish the X and the Y chromosomes from the autosomes of metaphase. All of the 40 chromosomes at metaphase are rod-shaped having no distinct constriction. The positions of the kinetochores are made distinguishable by the presence of minute second arms at their proximal ends. One may say that all of the 40 mouse chromosomes are telocentric chromosomes. In most chromosomes, a heteropycnotic area may still be recognized adjacent to the kinetochore region as is illustrated in Fig. 6.

On the basis of the above findings, the heterochromatic regions of the mouse chromosomes are presented in schematic fashion in Fig. 7. Each of the 38 autosomes has a small heterochromatic region adjacent to the kinetochore, while the X and the Y chromosomes appear to be entirely heterochromatic. These heterochromatic regions apparently persist into the inter-

phase nucleus as the chromocenters as evidenced in the Fig. 1 type of spermatogonial nuclei. The presence of fewer and larger chromocenters in the interphase nucleus of other mouse tissues can be explained as the aggregates of several heterochromatic regions. This may be analogous to the situation observed in the larval salivary gland cells of *Drosophila melanogaster* where heterochromatin from all the chromosome pairs is aggregated into one large chromocenter. It is tempting to make one further analogy from what is known in *Drosophila melanogaster*. In this insect the Y chromosome is almost entirely heterochromatic and, at the same time, there is only one known genetic locus for a morphological characteristic, although the Y chromosome has been shown to influence quantitative characters and male fertility [1]. The fact that only a very few well-established sex-linked morphological characters have been turned up in mice [10, 5, 6, 8, 7, 9, 19] may be a reflection of the almost entire heterochromatic condition of the X and Y chromosomes as observed at present.

DISCUSSION

As early as 1909, some observers (O. Rosenberg and J. B. Overton) were struck by the fact that in some cases, the vegetative cell nuclei of higher plants contain numerous karyosomes which approximate the chromosome number. These findings stimulated further studies to identify the karyosomes of the interphase nuclei with specific regions of the chromosomes. Accordingly, precise descriptions of heterochromatic regions of *Drosophila melanogaster* chromosomes have been made by Heitz [12] and Kaufmann [13]. Their findings were based on observations made upon ganglion cells of larvae. The present findings on mouse chromosomes suggest that by choosing suitable cells, heterochromatic regions may be identified in mammalian chromosomes.

In mammalian cytology, karyosomes or chromocenters are often confused with nucleoli or plasmosomes [4]. Caspersson's assumption that the nucleolus is produced by the chromocenter [2, 3] appears to be based upon his findings with *Drosophila melanogaster* [4]. Kaufmann's study of *Drosophila melanogaster* chromosomes [13], however, clearly demonstrates that the nucleolus is produced by the nucleolus organizers of the X and the Y chromosomes and the heterochromatic regions of the two pairs of autosomes are not concerned with nucleolus formation. Although the X and Y chromosomes of *Drosophila melanogaster* have heterochromatic regions, it is the secondary construction which is concerned with nucleolus formation. The present findings indicate that in the case of the mouse, too, the heterochromatic regions of the 38

autosomes play no role in nucleolus formation. Only the X and the Y are found in association with the nucleolus. Sachs [20] also describes the formation of the nucleolus in the mouse by the totally heteropycnotic X and Y during meiotic prophase. The nucleolus organizer of the X and the Y appears to be located extremely close to the respective kinetochore loci. Thus, they are not visualized as secondary constrictions in metaphase.

Inasmuch as the mouse X and Y are concerned with nucleolus formation, the relation of the X and the Y to the sex vesicle during meiotic prophase is strikingly different from that of the rat. In the case of the rat, nucleoli have been observed to be organized by a few autosomes [18]; thus, during meiotic prophase, the sex vesicle is organized independently of the nucleoli. The totally heteropycnotic X and Y lie embedded within the sex vesicle paired along the length of their homologous regions [16, 17]. In the case of the mouse, however, the sex vesicle is actually the nucleolus and the X and the Y are not enveloped by the vesicle, but they are connected end to end through the vesicle as evidenced in diplotene (Fig. 4). Thus, there appears to be no possibility of the exchange of genetic material between the mouse X and Y during meiotic prophase.

SUMMARY

The heterochromatic regions of mouse spermatogonial chromosomes have been described. The 38 autosomes have heterochromatic regions adjacent to the kinetochore loci. The X and the Y chromosomes are entirely heterochromatic and are concerned with nucleolus formation.

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ANALYSIS OF THE SURFACE STRUCTURES OF THE SEA URCHIN EGG BY MEANS OF ANTIBODIES[1]

I. COMPARATIVE STUDY OF THE EFFECTS OF VARIOUS ANTISERA

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Received March 28, 1957

Injection of cell or tissue homogenates into rabbits gives rise to the appearance in the serum of a large number of antibodies against the antigenic material injected. An antiserum, so obtained, may exhibit a toxic action when added to a suspension of living cells of the type used as a source of antigens. The effects of antisera on cells are due, ultimately, to reactions between the antibodies in the antisera and the antigens in the cells. Since these reactions are characterized by a high degree of specificity, the action of antisera may constitute a valuable tool for the study of cell structures. By this means, it has been possible, for example, to analyze the surface structures of certain bacteria in considerable detail [32]. However, owing to the complexity of the response induced in the treated cells, the resolution of a "cytotoxic" action into its components may be extremely difficult. For review and references cf. [17].

The sea urchin egg, when treated with antiserum, does not behave in an exceptional way. When exposing a relatively small number of eggs to an antiserum obtained from rabbits, several changes are induced in the eggs and can be recorded microscopically. Among these changes, coagulation of the jelly layer of the eggs, and parthenogenetic activation are of particular interest [21, 22]. Increasing the number of eggs per volume antiserum gradually leads to complete disappearance of the microscopic response. However, irreversible alterations will be found in such eggs upon fertilization after removal of the antiserum. Among the manifestations of the antiserum action recorded in this way are depression of the rate of fertilization [24], disturbances of the cortical reactions in the fertilized eggs, induced polyspermy, and various anomalies of development [25].

Most of the response reactions are physiologically complex. However, there are several reasons which make the sea urchin egg exceptionally well

[1] Supported by grants from the Swedish Natural Science Research Council.

suited for this type of immunological analysis. Thus, the cell surface of the egg shows a high degree of organization, exhibiting a number of specific functions in fertilization and cell division. Hence, by interfering with these functions, the antibodies will give rise to a spectrum of distinct injuries of the eggs. These injuries can be used for recording the different actions of antiserum. Furthermore, the cells are sufficiently large for microscopic recording of the number of eggs, affected in various ways by a given amount of antiserum. This makes it possible to introduce quantitative methods as a prerequisite of a closer analysis. On the other hand, if the cells were larger, the use of such test methods would become impracticable. Finally, the sea urchin egg is well known from both the chemical and the physiological viewpoint, and the immunological analysis can therefore be carried out in combination with several other methods.

The species- and tissue specificity of the reactions evoked by antiserum have been demonstrated previously. It has also been shown that several of these reactions can be recorded quantitatively. In this paper additional data will be given, bearing on the dose-response relationships and on several variables governing the behavior of the eggs exposed to antiserum. The potencies of six different antisera have been compared in greater detail. The results of these comparisons led to the formulation of certain hypothetic conclusions concerning the occurrence of a number of antigenic factors present in the surface layers of the egg.

MATERIAL AND METHODS

The experiments were performed at the Stazione Zoologica of Naples, using eggs of the sea urchin *Paracentrotus lividus* as the main object of study.

Antisera were prepared by injecting rabbits, weighing about 3 kg, with unfertilized sea urchin eggs, sperm and isolated egg jelly. For control of activity on the eggs, samples of serum were withdrawn from the rabbits prior to injection. All these controls were negative. Two serum pools, each obtained from 5 untreated rabbits, were used as controls.

The number of the *anti Paracentrotus sera* used and the mode of their preparation are given in Table I. Each antiserum was the serum pool of 3-5 rabbits. The *anti egg sera* were obtained by injecting unfertilized eggs, deprived of their jelly layers at pH 5.2 [42]. Dialyzed and lyophilized egg homogenates were injected as antigen sources, using *different batches of eggs for each antiserum. Anti jelly serum J-3* was prepared by injecting jelly substance isolated at pH 5.2 and *purified by repeated precipitation with ethanol* [42]. *J-4* was obtained by injecting an *unpurified* material, isolated in the same way, but simply concentrated by pervaporation. In both cases the material was dialyzed against distilled water and lyophilized before injection.

TABLE I

*Mode of preparation of anti-*Paracentrotus *sera.*
For details see text.

Antiserum	Number of rabbits used for serum pool	Antigen used for injection	Method of injection[a]	Amount of material injected/rabbit[b]
P-9	5	jelly free eggs	presens.	119 mg N ($\sim 8.5 \times 10^6$)
P-10	5	jelly free eggs	presens.	103 mg N ($\sim 7.5 \times 10^6$)
P-11	3	jelly free eggs	adj.	43 mg N ($\sim 3.2 \times 10^6$)
J-3	3	jelly substance purified	adj.	220 mg fucose ($\sim 15 \times 10^6$)
J-4	3	jelly substance not purified	adj.	60 mg fucose ($\sim 4 \times 10^6$)
Sp-6	5	sperm, washed	presens.	96 mg N (unknown)

[a] presens. = Presensitization; adj. = Adjuvant; for details cf. [22].
[b] As total nitrogen (eggs and sperm) or fucose (jelly substance) actually injected. The figures in brackets give the approximate number of eggs to which these values correspond.

The *anti sperm serum* was obtained by injecting large amounts of washed sperm which had been dialyzed, homogenized and lyophilized. In addition to the *anti Paracentrotus sera* described in Table I, one antiserum against unfertilized eggs of *Psammechinus microtuberculatus* and one against those of *Arbacia lixula* (5 rabbits each), were used as controls for the species specificity of the reactions. Both were prepared in the same way as the *anti Paracentrotus sera.* The methods used for the injections of the rabbits and the further treatment of the sera have been presented in a previous article [22].

The *eggs* used in the experiments were obtained from the sea urchins according to Harding and Harding [7]. The preparation of the material for the immunological experiments has been described earlier [22]. For the determination of the number of eggs in a sample, aliquots were pipetted into a measuring cylinder and diluted to contain 300–400 eggs per 0.1 ml. Three to five aliquots of 0.1 ml of the diluted suspension were then transferred to checkered slides and all the eggs in each aliquot were counted in the microscope. Calibrated pipettes were used for all transfers. On the basis of these counts, the initial suspension was diluted to give the concentration of the eggs wanted in the final test. The method is rapid and yet sufficiently accurate for the present purpose.

The *jelly precipitating* and the *activating* effects of the antisera were tested in experiments performed as described previously [22]. In addition, the procedure is outlined schematically in Fig. 1 and further details will be given in the text below.

Antiserum treatment of the eggs for the study of *inhibition of fertilization* was carried out according to the method given in a previous communication [25]. The sperm used for fertilization was diluted immediately before insemination, to give a final dilution

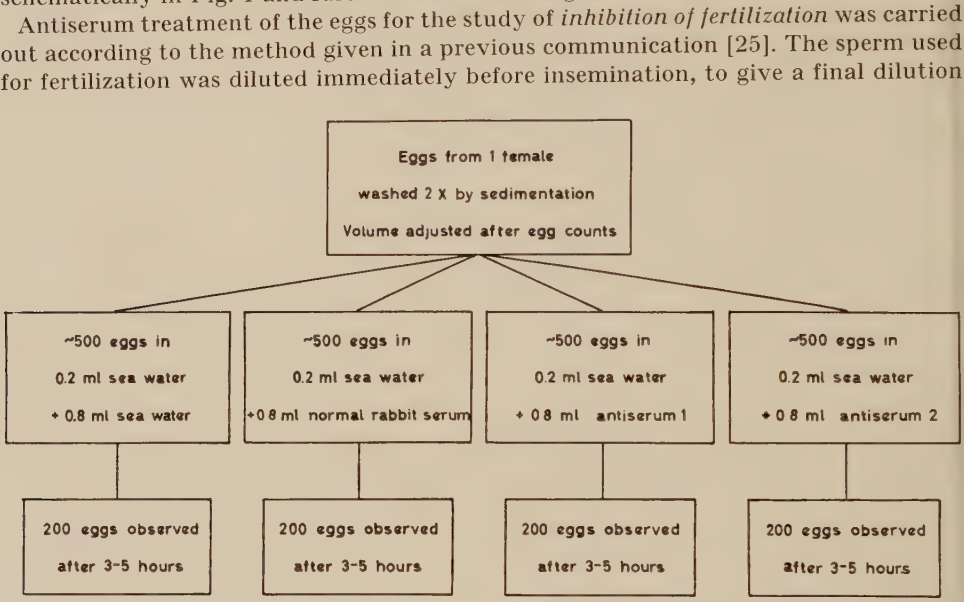

Fig. 1.—Typical procedure for testing the jelly precipitating and activating actions of two antisera.

of 1×10^6 spermatozoa per ml egg sperm mixture. All inseminations within one experiment were made with the sperm from the same suspension and as simultaneously as possible. The *rate of fertilization* was determined according to Hagström and Hagström [6]. In all experiments one aliquot of each egg sperm mixture was kept in the sea water for the determination of *fertilizability* and *cortical inhibition of the eggs*, and for *control of development*, respectively. The procedure is illustrated in Fig. 2.

The rate of fertilization can be defined as the *half fertilization period*, *F T 50*, i.e. the time needed for the fertilization of 50 per cent of all the fertilizable eggs in an experiment. This value can be obtained by transforming the percentages of fertilized eggs in an experiment into probits, and calculating the FT 50 from the probit/log time relationship [9, 10]. In this study and the subsequent papers the probit regression lines were calculated by applying the *maximum likelihood transformation*, after fitting a provisional line to the experimental values [1, 2]. One calculation was usually sufficient. Linearity of regression was tested by a χ^2-test [1]. Experiments yielding values with unsatisfactory linearity were discarded. As a rule, the linearity of the probit regression lines obtained with eggs pretreated with serum was rather good. The FT 50 was then computed from the regression equation. As an estimate of the error involved in the FT 50 values the 95 per cent confidence limits were calculated [1]. All points constituting a probit line within a fertilization rate experiment were

derived from the same population of antiserum treated and fertilized eggs (cf. Fig. 2). Hence, the errors involved in duplicate antiserum treatments and inseminations were not included as sources of variability in the FT 50 values or in their confidence limits. The main sources of error, however, consisted in the time necessary for killing the sperm and the decision as to whether or not the antiserum pretreated eggs actually had been fertilized. The duplicate experiments, therefore, gave always practically

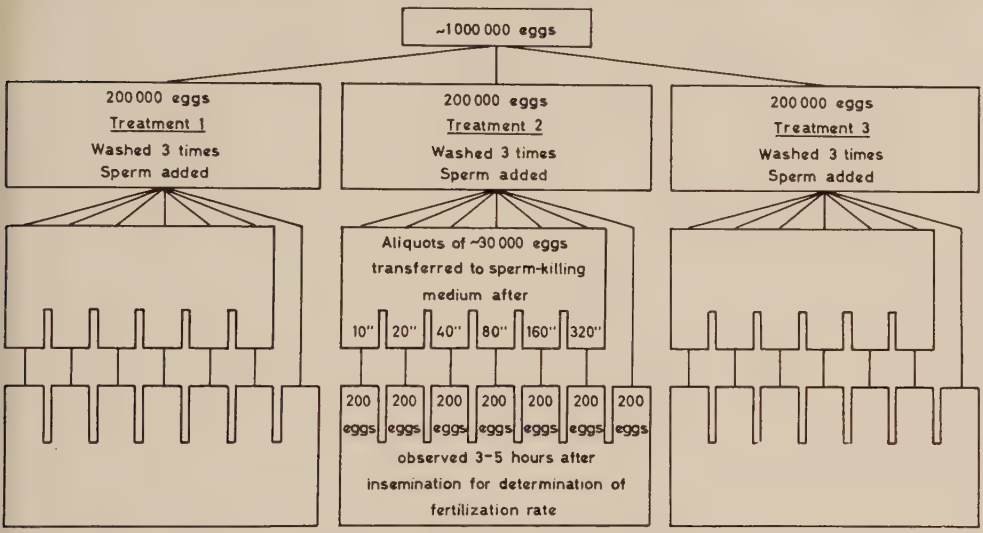

Fig. 2.—Typical procedure for the determination of the inhibitory effects of antiserum on fertilization. Eggs from 1 female pretreated with normal serum (treatment 1) and with two different antisera (treatments 2 and 3). The total amount of serum used for the treatments, the total volume of the reaction mixtures and the number of eggs in these mixtures were the same in all cases. Egg numbers were determined accurately. The rate of fertilization was determined on the aliquots transferred to the sperm killing medium. The remaining aliquots were used for determination of *fertilizability*, of *cortical damage* and for *control of development*.

identical FT 50 values and this procedure can be considered sufficient for the present purpose.

The *precipitin content* of the antisera was tested by a modification of the Ouchterlony technique [18], as described elsewhere [23]. Here, undialyzed antisera were used. In addition, *the sperm agglutinating potency* of the antisera was tested. A detailed description of the procedure will be given in a forthcoming paper.

The various effects of the *anti Paracentrotus sera* were species-specific. The results of the specificity controls were similar to those described earlier and are not included in this paper.

Precipitation of the Jelly Layer

The jelly precipitating action of antiserum and the specificity of this reaction have been described elsewhere [19, 21, 22]. Summarizing: in affected

TABLE II

Jelly precipitating (a) and activating action (b) of the antisera, studied in individual tests on the eggs from 55 females.

Aliquots of 500 eggs in 0.2 ml sea water, mixed with 0.8 ml of various antisera or with normal serum. Observation of *jelly precipitation* after 120 minutes at 20°C. Observation of *activation* after ~ 180 minutes in separate aliquots, transferred to the cold room for 2 hours immediately after mixing of eggs and sera. Cf. text.

C = normal serum.

Antiserum	(*a*) Number of females with eggs responding to jelly precipitating action	(*b*) Number of females with eggs responding to activating action
P-9	15	27
P-10	11	25
P-11	8	38
J-3	27	3
J-4	29	31
Sp-6	0	1
C	0	1

eggs the jelly layer, normally invisible, becomes visible in the microscope and, in the case of a strong reaction, contracts irreversibly. Jelly precipitated eggs are usually agglutinated. Even with the most potent antisera applied thus far, a great excess of antiserum is needed to obtain a response. The reactivity of the eggs varies during the course of the spawning season and can be correlated with the fertilizability of the eggs.

The response of the eggs is graded. The degree of a reaction is characterized by (1) the number of eggs with visibly precipitated jelly layers, (2) the degree of jelly contraction and (3) the extent of agglutination. Since these three characteristic features never showed an independent variation from each other, the number of visibly affected eggs could be used as a convenient, though not very sensitive index of the potency of the different antisera.

In Table II*a* the results are listed of individual tests with six *anti Paracentrotus sera* on the eggs of 55 females. It is apparent that the two *anti jelly sera* affected the eggs from considerably more females than did the *anti egg sera*. The *anti sperm serum* was inactive. Since the *anti egg sera* only affected eggs from those batches which also responded to *J-3* and *J-4*, a more detailed picture could be obtained by pooling the results of the 29 cases which responded positively to *J-4* (Fig. 3*a*). The differences between the *anti jelly sera* on the one hand, and the *anti egg sera* on the other, were statistically

significant. Numerous attempts to induce reactions with *J-3* and *J-4* in inert batches by improving the experimental conditions [22] were without success. Hence, we may conclude that eggs not responding to *J-3* and *J-4* are *non-reactive*.

As shown by the typical results in Table III, the two *anti jelly sera* did not differ much in potency. Exposing fully reactive eggs to any of the three

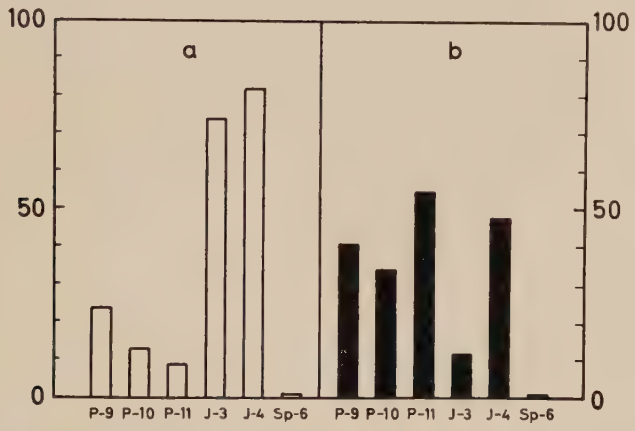

Fig. 3.—(*a*) Pooled numbers of jelly precipitated eggs from the 29 females responding positively to antiserum J-4 according to Table II*a* and (*b*) pooled numbers of activated eggs from the 38 females responding positively to antiserum P-11 according to Table II*b*. For details cf. Table II. Values are expressed as per cents of total counts of 5800 eggs (200 eggs/female) in each antiserum in Fig. 3*a* and of 7600 eggs (200 eggs/female) in each antiserum in Fig. 3*b*.

TABLE III

Jelly precipitating action of anti jelly sera J-3 and J-4, tested in varying dilutions on fully responsive eggs from 5 females.

500 eggs in 0.2 ml sea water added to 0.8 ml antiserum–sea water mixtures. Observation after 120 minutes at 20°C. 200 eggs counted in each case.

Final concentration of antiserum in test (% v/v)	Per cent jelly precipitated eggs Female No. 1		2		3		4		5	
	J-3	J-4	J-3	J-4	J-3	J-4	J-3	J-4	J-3	J-4
5	0	0	0	0	0	0	0	0	0	0
10	0	0	5	5	5	5	0	5	0	0
20	15	25	90	90	50	55	0	80	60	95
40	100	100	90	90	100	100	50	100	100	100
80	100	100	90	90	100	100	100	100	100	100

anti egg sera did not always yield a 100 per cent response even with large excesses of antiserum. Moreover, the slope of the response curve seemed to be less steep than that obtained with *J-3* or *J-4*. The jelly precipitating action of *P-9*, *P-10* or *P-11* was probably, at least in part, due to the action of cross-reacting antibodies.

While the *anti jelly sera* represented relatively simple immunological systems, the *anti egg sera* were much more complex (cf. Fig. 9). A number of experiments were made in order to find out whether the low activity of the *anti egg sera* was due to the presence in them of various kinds of inhibiting antibodies. Thus, the eggs were either pretreated with *anti egg serum* before they were treated with *anti jelly serum*, or they were exposed to mixtures of *anti egg* and *anti jelly serum*. Thus far nothing could be found that suggested the presence in the *anti egg sera* of inhibiting antibodies.

Activation

As described previously, antiserum may exert a species- and tissue-specific activating action on the unfertilized egg [21, 22]. An irregular fertilization membrane, nuclear activation and monaster formation, sometimes followed by cell division, are characteristic features of the eggs activated by antiserum.

Activation represents a highly complicated physiological response of the egg cell. Hence, the extent of response obtained with a given amount of antiserum will be influenced by a great number of variables. As in the case of the jelly precipitation, *the state of maturity of the eggs* certainly plays an important role. There will always be found eggs which cannot be activated, no matter how favorable the experimental conditions are. In addition it has been shown that the *jelly layers* may inhibit the response. In general, jelly free eggs respond better than normal eggs. On the other hand, removal of the jelly layers *at too low a pH*, i.e. below 5, may also have an inhibitory effect [22].

The influence of two additional variables is illustrated with the aid of the data given in Table IV. It is apparent that *aging of the eggs* after isolation lowered their reactivity. This effect was usually not observed until one hour after removal of the eggs from the sea urchins. It should be emphasized that the aged eggs used in the experiments, whose results are listed in Table IV, were fully fertilizable. On the other hand, a considerable improvement of the response could be obtained when the experiment was carried out *in the cold* (4°C). The data of Table IV are typical for all activating antisera used in this study. It should be noted that there existed a number of egg

TABLE IV

Activating action of antiserum under various conditions, studied in individual tests on the eggs from 14 females.

Aliquots of 500 eggs in 0.2 ml sea water, mixed with 0.8 ml of anti egg serum P-10 or with 0.8 ml of normal serum:

(A) Within one hour after isolation of the eggs from the sea urchins. Samples left at 20°C during the experiment.
(B) Four hours after isolation of the eggs. Samples left at 20°C during the experiment.
(C) Within one hour after isolation of the eggs. Samples transferred to the cold room (4°C) for two hours immediately after mixing of eggs and serum.

Observation of 200 eggs in each mixture 4–5 hours after the onset of the experiment.
AS = antiserum P-10, C = normal serum.

Female No.	Per cent activated eggs (A) AS	(A) C	(B) AS	(B) C	(C) AS	(C) C
1	0	0	0	0	0	0
2	5	0	<1	0	10	0
3	0	0	<1	0	32	0
4	<1	0	<1	0	60	7
5	24	0	36	0	81	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	40	0	0	0	89	0
9	8	0	2	0	42	0
10	5	0	<1	0	60	0
11	31	0	<1	0	35	5
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0

samples which could not be activated even when treated in the cold. No improvement was obtained when the eggs were transferred to the cold 30 minutes or later after mixing with the antiserum.

The improvement of the reactivity of the eggs at low temperature is probably not restricted to antiserum as the activating agent. More likely, it is the expression of a general tendency of the eggs to respond more readily to all kinds of activating stimuli at low temperature. For references cf. ([8], p. 198). However, it should be emphasized that the significant differences found at 20°C, between the actions of homologous antiserum, heterologous antiserum and normal serum, were also obtained in the experiments at 4°C.

Activation of the eggs at low temperature led to a complete separation of the

TABLE V

Activating action of anti egg and anti jelly sera, studied in individual tests on the eggs from 10 females, all responding positively to antiserum P-11.

Aliquots of 500 eggs in 0.2 ml sea water, mixed with 0.8 ml of various antisera, transferred to the cold room for 2 hours immediately after mixing. Observation of 200 eggs in each case after further 2–4 hours at 20°C.

C = normal serum.

Anti-serum	Per cent activated eggs Female No.									
	1	2	3	4	5	6	7	8	9	10
P-9	10	85	36	7	89	38	8	4	42	62
P-10	3	81	26	0	68	25	2	0	53	54
P-11	11	93	42	5	100	62	17	13	80	90
J-3	0	36	1	0	25	6	0	0	18	29
J-4	11	90	36	0	91	40	11	1	56	71
C	0	0	0	0	0	0	0	0	0	0

cortical and the nuclear activation from cell division. Thus, eggs activated at 4°C showed membrane formation, migration of the nucleus to the center of the cell, and monaster formation as usual, but the nuclear and cell division were completely inhibited. However, when transferred from the cold to room temperature even after several hours, the activated eggs very often started to cleave.

The *average activating capacity of the different antisera* is shown in Table II*b*. Since the eggs which responded to all antisera were from the same females, a more detailed picture could again be obtained by pooling the results from those 38 cases in which the eggs responded positively to the strongest antiserum, *P-11* (Fig. 3*b*). Numerous attempts to bring about a response to *P-11* in inert egg batches, by using fewer eggs and/or more antiserum [22], were unsuccessful. The high variability of the extent of response among reactive egg batches is further illustrated by the data given in Table V.

The results show that all antisera except the *anti sperm serum* and the serum against the purified jelly substance, *J-3*, activated a considerable portion of the egg material. *P-11* was strongest, followed by *J-4* and *P-9*. *P-10* was the weakest of the three *anti egg sera*. The differences in potency of these sera are statistically significant; moreover, they held over the whole range of antiserum concentrations and, in fact, the response curves seemed to run parallel in all cases. Fig. 4 gives the results of a typical experiment where eggs from a *highly reactive batch* were tested in dilution series of all the five

activating antisera. The results suggest that the activating mechanism is the same for all cases.

In a series of experiments the eggs were exposed to mixtures of the weak antiserum *J-3* with *P-10* or *P-11*. The number of activated eggs obtained in these mixtures was always very similar to that obtained in corresponding

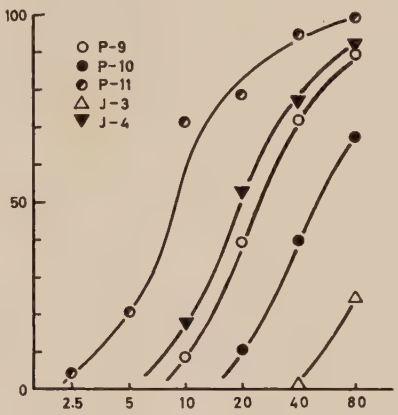

Fig. 4.—The activating effects of dilution series of the various antisera on the eggs from a highly reactive female. For details of treatment cf. Table V. *Abscissa,* final concentration of antiserum (per cent v/v). *Ordinate,* per cent activated eggs. Values based on counts of 200 eggs in each case.

mixtures of *P-10* or *P-11* with normal serum. This indicates that the low activity of *J-3* was not due to the presence, in this serum, of inhibiting antibodies.

The eggs activated in the different antisera behaved rather differently. Thus, *P-9* and *P-10* always led to the formation of heavily precipitated membranes, i.e. strongly light-refracting, or large membrane blisters and a strongly inhibited breakdown of the cortical granules. The activated eggs almost always underwent a number of cell divisions. In *P-11* and *J-4*, the disturbances of the cortical reactions of the eggs were not at all marked and the membranes appeared thinner. In contrast, the membrane blisters formed were rather small. Moreover, the activated eggs cleaved only rarely and the activation was usually arrested at the stage of the dissolution of the nuclear membrane. The eggs then slowly cytolyzed.

Depression of the Rate of Fertilization

When an egg sample is mixed with sperm and aliquots of the mixture are transferred to a medium which kills the spermatozoa but not the eggs, one can measure the "rate of fertilization" by determining the proportion of the eggs fertilized at various time intervals of the egg-sperm interaction [6, 27]. Pretreatment of the eggs with *anti egg serum* reduces the rate of fertilization.

The action of antiserum is species-specific. It is due to an interaction of antibodies with the egg, rather than with the sperm [24].

Eggs fertilized after pretreatment with antiserum often do not develop; they show more or less pronounced anomalies in their cortical reactions. Therefore, in determining the fertilization rate, the eggs were classified as

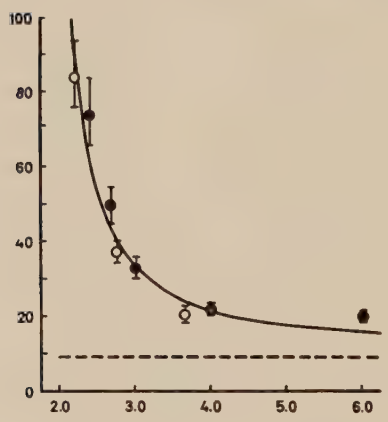

Fig. 5.

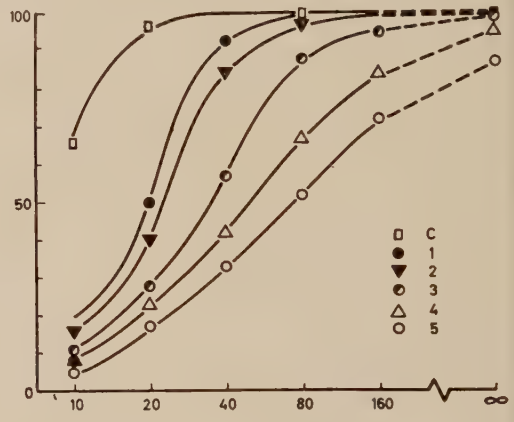

Fig. 6.

Fig. 5.—Fertilization rate of eggs pretreated with anti egg serum P-10 in varying concentrations. Dots and circles represent two independent experiments with gametes from different sea urchins. Broken line: FT 50 values obtained with eggs pretreated with normal serum.
Abscissa, egg/antiserum ratios $\times 10^{-5}$. *Ordinate*, FT 50 values in seconds, with 95 per cent confidence limits.

Fig. 6.—Fertilization rate of eggs pretreated with anti egg serum P-10 in varying concentrations. Aliquots of 120,000 eggs were used for treatment. Serum mixtures used for pretreatment: *C*, normal serum, 0.5 ml; *1*, 0.2 ml P-10 + 0.3 ml *C*; *2*, 0.3 ml P-10 + 0.2 ml *C*; *3*, 0.4 ml P-10 + 0.1 ml *C*; *4*, 0.45 ml P-10 + 0.05 ml *C*; *5*, 0.5 ml P-10.
Abscissa, time in seconds of egg-sperm interaction. *Ordinate*, per cent fertilized eggs. Values based on counts of 200 eggs in each case.

fertilized *whenever they became activated by the sperm, with no regard taken to ensuing cell cleavage and development.*

The rate of fertilization of untreated eggs varies widely with different batches of gametes [10, 19]. The variability is considerably less, however, if the eggs are pretreated with normal rabbit serum. This treatment apparently "normalizes" the state of the eggs. Under the conditions prevailing in the experiments approximately the same maximum rate of 5–10 seconds was obtained. When the normal serum is replaced by a mixture of antiserum and normal serum, the fertilization rate is depressed as compared with the normal serum control, but the variability of the rate is still less than that with untreated eggs.

The fertilization rate varies inversely with the amount of antiserum used per egg. For illustration, in Fig. 5, the FT 50 values, obtained with two dilution series of antiserum *P-10* in two independent experiments, are plotted against the egg/antiserum ratios. (Number of eggs in test divided by ml antiserum used. Cf. [25].) As can be seen, the FT 50 values change rather abruptly within a narrow range of the egg/antiserum ratio, and approach infinity below a certain critical ratio. More than 90 per cent of the eggs were fertilized at all the higher egg/antiserum ratios, whereas the fertilizability of the eggs was rather low *below* the critical value. Moreover, it should be noted that the final decrease of the FT 50 values to that of the control is slow and asymptotical over a wide range of antiserum concentrations. This type of exponential response was typical for normal eggs and for all inhibiting antisera. Jelly free eggs behaved differently [24].

At higher antiserum concentrations, i.e. lower egg/antiserum ratios, the enhancement of the FT 50 was always connected with a decrease of the slope of the fertilization curve (Fig. 6). It is not clear whether this was also the case when low amounts of antiserum were used. In fact, certain results indicate that the increase of the FT 50 values under these conditions may be due to an increase of a time lag period. At present, no conclusions can be drawn concerning the possible mechanism of antiserum action in these experiments, since the fertilization rate is a function not only of the state of the eggs but also of the fertilizing capacity of the sperm. At the great dilution used in our egg-sperm mixtures, this capacity decreases within very few minutes [27] and thus influences the fertilization rate, particularly in the case of eggs treated heavily with antiserum. It is essential that all the inhibiting antisera of this study acted in an identical manner.

In Table VI the results of two typical experiments are given, in which the fertilization rate-reducing potencies of the different antisera are compared. Here, the amount of antiserum per egg was low and 100 per cent fertilization was obtained in all cases. Nevertheless, the reduction of the fertilization rate obtained with the three *anti egg sera*, and the differences in their potencies, are significant. Fig. 7 shows the results of a typical experiment, in which the egg/antiserum ratio was lower. The concentrations used in the case of the three *anti egg sera* were not far from the critical levels at which fertilization is suppressed entirely. The weak antisera *Sp-6*, *J-3* and *J-4* were also tested at very high concentrations. At the highest concentration tested (egg/antiserum ratio $\sim 5 \times 10^4$), *Sp-6* and *J-3* did not reduce significantly the rate of fertilization. At this ratio, *J-4* was close to the level where it completely inhibits fertilization. Thus far, no correlation could be ascertained

TABLE VI

Depressing action of the various antisera on the rate of fertilization, studied in two independent experiments.

For experimental details cf. "Material and Methods". FT 50 values and 95 per cent confidence limits for the three anti egg sera calculated as indicated in the text, the values for the other sera estimated graphically. All values in seconds.

Egg/antiserum ratio: Number of eggs divided by ml of antiserum used in test. Total volume of reaction mixture 1.5 ml.

C = normal serum.

	Experiment 1 Egg/antiserum ratio: 3.94×10^5		Experiment 2 Egg/antiserum ratio: 3.99×10^5	
Antiserum	FT 50	95 % confidence limits	FT 50	95 % confidence limits
P-9	26.4	23.9–29.3	38.6	36.2–41.3
P-10	10.0	8.7–11.3	19.6	18.2–21.0
P-11	19.2	17.2–21.4	27.7	25.9–29.6
J-3	~5	—	~10	—
J-4	~5	—	~10	—
Sp-6	~5	—	~10	—
C	~5	—	~10	—

between the fertilization rate depressing potencies of *J-3* and *J-4* and the precipitabilities of the jelly layers of the fertilized eggs.

It can be concluded from these data that the two *anti jelly sera* possessed a very weak fertilization rate-depressing activity when compared with the *anti egg sera*. The activity of the serum against the purified jelly preparation is hardly noticeable. The *anti sperm serum* was inactive. Although the determination of relative potencies was beyond the scope of this study we may, however, state that the three *anti egg sera* differed significantly in their potency, *P-9* being the strongest, *P-10* the weakest, and *P-11* of intermediate nature.

Only a few experiments of this type have thus far been made on jelly free eggs. It may be mentioned that the antisera *J-3* and *J-4* appeared to be as inactive on jelly free eggs as they were on normal eggs.

Cortical Damage

Certain characteristic anomalies in the cortical reaction are among the most striking features appearing in eggs fertilized after pretreatment with

antiserum. The eggs do not form complete fertilization membranes but rather membrane blisters of different sizes. The membranes are strongly light-refracting. The cortical granules often seem to be precipitated and do not dissolve normally. These and other related effects of antiserum have been described previously [25].

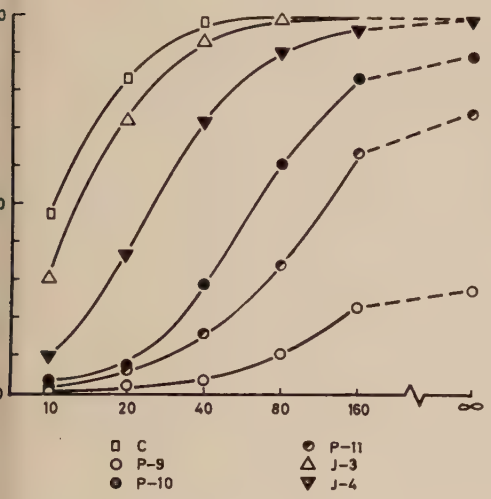

Fig. 7.

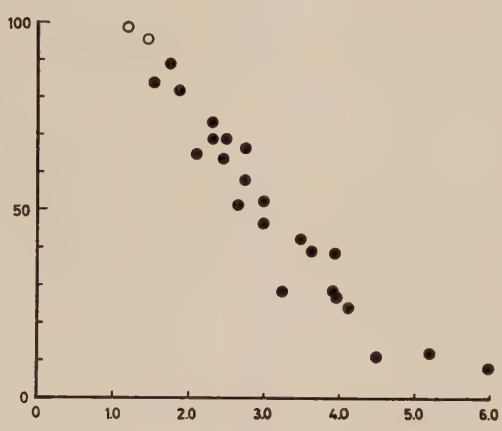

Fig. 8.

Fig. 7.—Fertilization rate of eggs pretreated with various antisera. Egg/antiserum ratios 2.3×10^5 in all cases.
Abscissa, time in seconds of egg-sperm interaction. *Ordinate*, per cent fertilized eggs. Values based on counts of 200 eggs in each case.
Fig. 8.—Cortex-damaging effects of pretreatment with anti egg serum P-10 in varying concentrations. Each dot and circle represent an independent experiment with gametes from different sea urchins.
Abscissa, egg/antiserum ratios $\times 10^{-5}$. *Ordinate*, per cent eggs with cortical damages. Values based on observation of 200 *fertilized* eggs in all cases. More than 90 per cent of the eggs were fertilized in all experiments represented by dots. At the two lowest egg/antiserum ratios (circles), only ~30 per cent of the eggs were fertilized.

Although the response of the eggs was graded, the number of visibly affected eggs proved to be a good index of the antiserum activity. Fig. 8 shows the results of 25 independent experiments in which the percentage of affected eggs is plotted as a function of the egg/antiserum ratio. As can be seen, this percentage was approximately inversely proportional to the egg/antiserum ratio over a relatively wide range of antiserum concentrations. In view of the fact that the points in Fig. 8 represent values obtained with eggs and spermatozoa of different origin and with different antiserum treatments, the deviations from the proportionality are small.

TABLE VII

Cortex-damaging effect of the various antisera, studied in five independent experiments.

For experimental details cf. "Material and Methods".
Egg/antiserum ratio: Number of eggs divided by ml of antiserum used in test. Total volume of reaction mixture 1.5 ml. Total amount of serum constant in all experiments (1.0 ml).
Observation of 200 eggs in each mixure 8–10 hours after fertilization.
A, per cent unfertilized eggs.
B, per cent eggs with cortical damage, figures based on fertilized eggs only.
C = normal serum; ? = classification uncertain, cf. text.

	Egg/antiserum ratios									
	1.2×10^5		2.3×10^5		3.0×10^5		3.6×10^5		4.3×10^5	
Antiserum	A	B	A	B	A	B	A	B	A	B
P-9	96	—	75	—	5	50	7	27	3	16
P-10	98	—	10	70	2	58	3	36	0	19
P-11	~100	—	23	10?	2	19?	1	14?	0	3?
J-3	3	0	0	3?	0	0	0	0	0	0
J-4	0	14?	0	4?	0	5?	0	10?	0	5?
Sp-6	0	5	0	3	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0

The differences between the potencies of the five antisera are illustrated by the data given in Table VII. Striking differences are apparent between the *anti egg sera*, on the one hand, and the *anti jelly sera* and *anti sperm serum*, on the other. Moreover, while the potencies of *P-9* and *P-10* were approximately equal, that of *P-11* was significantly lower. In addition, the eggs classified as damaged after pretreatment with *P-11* had a different appearance than those treated with *P-9* and *P-10*; *P-11* affected the membrane formation much less, and inhibited mainly cytoplasmatic cleavage. Similar effects were obtained when *J-4* was applied in very high concentrations. It might well be that the injurious effects obtained with *P-11* and *J-4* were quite unrelated to those obtained with *P-9* and *P-10*. It should also be recalled that the cortical damage was much less pronounced in eggs activated in *P-11* and *J-4* than in those activated in *P-9* and *P-10*.

The same differences in potencies between the various antisera also prevailed when they were tested on jelly free eggs.

Other Activities of the Antisera

The response of antiserum pretreated eggs upon fertilization shifts within a rather narrow range of the egg/antiserum ratio from 100 per cent *fertili-*

zability to complete unfertilizability. Comparison of the different antisera showed a close correlation between their fertilization rate-depressing potency and their ability to render the eggs "unfertilizable". The mechanism of this antiserum activity has not yet been studied. Obviously, antibody action may render the eggs completely unsusceptible to the spermatozoa. However, it has to be considered that the occurrence of "unfertilizable" eggs in our experiments may be due to the unstable fertilizing capacity of the spermatozoa. In most cases, the percentage of unfertilized eggs would probably have decreased upon reinsemination.

In a previous note it has been described that pretreatment with antiserum renders the eggs susceptible to *polyspermy* [25]. One can assume that a close correlation between the polyspermy-inducing and the cortex-damaging potencies of the antisera exists. Unfortunately, it has not yet been possible to test this assumption. It can easily be understood that a comparison of the potency of *P-9* or *P-10* with that of *P-11* would be of particular interest.

Antiserum pretreated eggs which showed the cortical injuries on fertilization practically never developed beyond hatching. Often they did not divide at all. However, there are certain indications suggesting the occurrence of an independent antiserum activity which only inhibits *cytoplasmatic cleavage* (cf. the properties of *P-11* and *J-4*, described above). Mild antiserum treatment of the eggs caused a general *retardation of cleavage* in the developing egg. None of these effects has yet been studied, nor has the reversible *wrinkling effect* of antiserum on the surface of the unfertilized egg been investigated, owing to the difficulties in obtaining quantitative records on eggs with intact jelly layers.

In a special series of experiments the *sperm-agglutinating* potencies of all antisera were compared. The experimental details and results will be described later. It should be mentioned here that all three *anti egg sera*, as well as the *anti sperm serum*, acted as agglutinants. The activities of *J-3* and *J-4* were very low. The action of *Sp-6*, however, was much more complex than that of the *anti egg sera*, and its potency was about 100 times higher. The sperm-agglutinating actions of the antisera were less species-specific than their actions on the eggs.

The *precipitin content* of the antisera was tested qualitatively, using the techniques of diffusion in saline agar described elsewhere [18, 23]. Figs. 9*a* and *b* are two typical illustrations of the reaction between the antisera and the soluble fraction of a concentrated egg homogenate. It should be noted that the *anti jelly serum J-3* was completely inactive, while *J-4* contained some antibodies against soluble egg-antigens [20]. The precipitation pattern

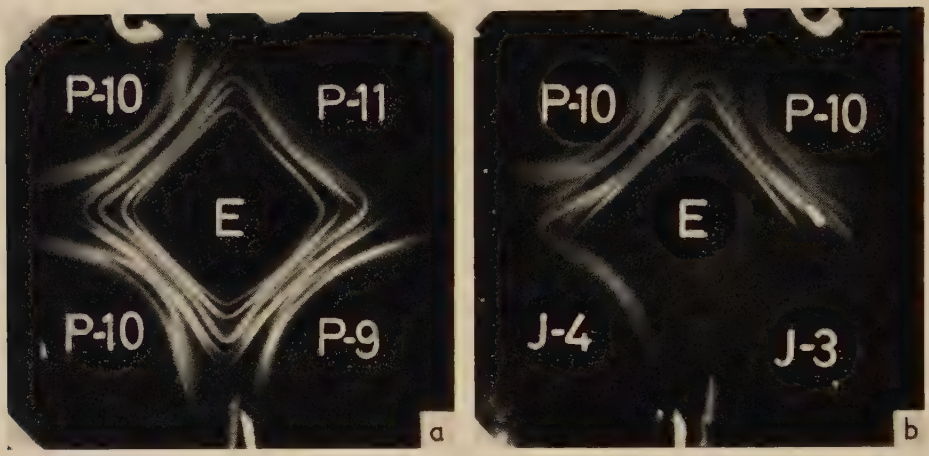

Fig. 9.—Precipitin tests in agar. Dark field photographs, taken after 2 days incubation at 38°C followed by 2 weeks in the refrigerator. *E*, soluble part of egg homogenate, diffusing from reservoir in the center. The various antisera diffused from the peripheral reservoirs.

of the *anti egg sera* was complex and, therefore, was not analyzed in detail. A point which is of interest is the apparent absence, from antiserum *P-11*, of several antibody fractions.

Discussion of Antiserum Action

Not including the jelly layer, the *Paracentrotus* egg has a surface area of 2.54×10^{-4} cm^2, an area which is ~ 150 times larger than that of the human red blood cell. Thus, it is not surprising that the amounts of antiserum needed for the experiments described here are appreciably larger than those usually employed in experiments with blood cells. Moreover, it may be assumed that the concentration of certain antibodies in our antisera is rather low, which is also indicated by the large amounts of antigenic material required for the preparation of potent antisera. This complicates quantitative work since, for technical reasons, the experiments have to be performed under conditions where both the egg numbers and the antiserum amount have to be considered. Nevertheless it has been demonstrated that the egg/antiserum ratio is an appropriate measure of the reactivity of the antiserum. It should be pointed out, however, that the range of dilution in which the relationships between egg response and egg/antiserum ratio hold has not yet been studied.

TABLE VIII

Comparison of potencies of the various antisera.
0 = inactive, ± to + + + = very weak to strongly active.

Antiserum	Activity			
	Jelly precipitation	Activation	Fertilization rate depression	Cortical damage
P-9	+	+ + ±	+ + +	+ + +
P-10	±	+ +	+ +	+ + ±
P-11	±	+ + +	+ + ±	+
J-3	+ + +	±	0?	0
J-4	+ + +	+ + ±	+	±
Sp-6	0	0	0	0

Considerably more work would be needed for the determination of the relative potencies of the various antisera with regard to their activities in precise quantitative terms. On the other hand, the data given in this paper bearing on the differences in potency between these antisera are significant. The data are summarized in Table VIII.

The antisera used in this study were serum pools from 3–5 animals, each animal within the same group having been injected with the same material. The variations in the antiserum potencies can most likely be ascribed to differences in the composition of the injected material, to the amounts injected, and to the route of injection. The differences encountered between the *anti sperm serum*, the *anti jelly sera* and the *anti egg sera*, respectively, were certainly due to differences in the antigenic composition of the injected substances. This is unlikely in the case of the three *anti egg sera*. Since the two antisera *P-9* and *P-10* were prepared in almost the same way and had similar activities, it seems probable that the discrepancy between these and *P-11* was mainly due to differences in the amounts injected and/or in the routes of injection.

The *anti sperm serum Sp-6* exhibited none of the activities shown by the other antisera, which suggests a tissue specificity of all the effects described. Moreover, it indicates that the sperm agglutinating action of the *anti sperm serum* is due to antibodies which are different from those acting on the egg.

Comparison of the *jelly precipitating* activities of the different antisera suggests that jelly precipitation is caused by antibodies against one or several antigens located exclusively or mainly in the jelly layer. The action of the *anti egg sera* used in this study may have been due to cross reactions or to low contents of that particular type of antibodies.

The *activating* potencies of the antisera are not correlated with their jelly precipitating potencies. This is in agreement with earlier observations [22]. Moreover, the results show that the jelly precipitating antibodies do not participate in the activating effect. It may be significant that it is the antiserum *J-4*, obtained with the *crude* jelly preparation, which is strongly active, whereas *J-3* is inactive. Thus, it appears that the crude jelly preparation contains contaminating egg antigens, whose antibodies would act as the agents inducing activation. This assumption is also supported by the higher degree of heterogeneity of the antiserum *J-4* as compared with *J-3*, when tested with egg homogenate in the precipitin test (Fig. 9*b*).

The essential question whether only one type of antibody causes activation in all cases cannot yet be answered conclusively. The high activity of *P-11* and *J-4* may be due to the lack of some inhibiting antibodies, occurring in *P-9* and *P-10*, although results as those shown in Fig. 4 would not favor such an explanation.

While the influence of inhibiting antibodies in this connection is still unclear, it seems probable that the *difference in appearance* between the eggs activated in *P-9* and *P-10* and those activated in *P-11* and *J-4* is due to the absence from the latter of antibodies with an inhibitory action on the cortical reaction and membrane formation. This is further supported by the fact that eggs fertilized after pretreatment with *P-11* and *J-4*, do not show the cortical damage that occurs after treatment with *P-9* and *P-10*.

When comparing the *fertilization rate-depressing* potency of the different antisera, the comparison of the *anti egg sera* alone indicates a positive correlation of their activation-inducing and fertilization rate-depressing potencies. However, none of the *anti jelly sera* exhibited such an activity, in spite of the high activating potency of antiserum *J-4*. This indicates a high degree of complexity of this particular response. It may be postulated that each antibody which reacts with an antigen of the egg surface can act as an inhibitor of fertilization *independently of whether or not the antigen involved participates in the fertilization process.* Apart from the obvious possibility of direct interference of an antibody with sperm receptor sites in the egg surface, any antigen antibody reaction in this area will probably lead to secondary alterations, such as changes in the electric charge and in the

state of hydration. Any physical change of this type is likely to inhibit the successful attachment of the fertilizing spermatozoon. Moreover, if one assumes that the *living* spermatozoon has to be attached to the egg for a certain period in order to achieve activation, any reaction which slows down the reactivity of the egg may find its expression in a lowered fertilization rate. It may be pointed out that such considerations are valid for many types of fertilization inhibition experiments, in which various chemical means are used for the treatment of the eggs. For a discussion of these problems the fundamental work of Rothschild and Swann should be consulted. (For references and discussion cf. [26].)

Thus, while the results of this study do not permit any conclusions concerning the mechanism of the action of antiserum, we may state, however, that the jelly precipitating antibodies play an insignificant role, even when added to the eggs in a very large excess. Since the strongly jelly precipitating antisera *J-3* and *J-4* also appear to be inert when tested on jelly free eggs, it seems unlikely that the antigen concerned with jelly precipitation is an element of the egg surface.

The two *anti jelly sera* have a weak depressing action on the rate of fertilization. This may be taken as an indication that the antigens involved in this reaction are firmly bound to the egg and contaminate only to a minor degree the isolated jelly preparations used in the injections.

The *cortex-damaging* effect of pretreatment with antiserum, obtained in fertilized eggs, is due to antibodies against one or several egg antigens, not present in the jelly layer. The jelly precipitating antibodies are inactive in this connection. Moreover, the potencies of the three *anti egg sera* to cause cortical injury appear to be unrelated to their activating potency and to that depressing the rate of fertilization. This, taken together with the low variability of the egg response as a function of the egg/antiserum ratio, suggests (1) that some particular antigen antibody reaction (or reactions) may be responsible for the events observed microscopically and (2) that the participating antigen(s) may constitute an essential and predominating component of the molecules involved in the cortical reaction.

Figs. 9*a* and *b* demonstrate that the antisera *P-11* and *J-4* lack some of the fractions of precipitating antibodies, which are present in *P-9* and *P-10*. It remains to be seen whether the antibodies which cause cortical damage are part of these fractions. At this stage, no explanation can be given why the eggs activated with *P-9* and *P-10* almost always underwent cell divisions while those activated with *P-11* and *J-4* did not.

Some Physiological Considerations

Reactions between complementary chemical structures, or, in other terms, antigen antibody-like reactions, are believed to be of importance in fertilization. While the idea of the occurrence of such a mechanism will probably be accepted by all workers in this field, the question as to the nature of the structures involved is the subject of considerable discussions.[1] These discussions center mainly around the possible physiological significance of "fertilizin" [14, 36] as the specific sperm-recepting substance of the egg. According to the "fertilizin theory", a fairly specific substance, fertilizin, may agglutinate spermatozoa, preferentially those of the homologous species. Since this mechanism is believed to be of an "immunological" nature, the occurrence of an antifertilizin in the spermatozoa had to be postulated. An antifertilizin is also believed to occur in the eggs. According to current views, the most important manifestations of the reactions between the fertilizin and antifertilizin are (1) a precipitation of the jelly layer of the eggs, obtained with certain sperm- or egg extracts, and (2) an agglutination of the spermatozoa by jelly substance or "egg water". (For references and discussions cf. [26, 28, 29, 34, 36].) It may be assumed that "immunological" reactions of this kind do occur, even if the experimental evidence thus far presented in their favor cannot be accepted as conclusive. Since sperm agglutination and jelly precipitation can both be brought about by a variety of unrelated physical and chemical means, a distinction between "specific" and "non-specific" reactions becomes extremely difficult. A further discussion of these reactions falls beyond the scope of this paper.

The actual bearing of the fertilizin-antifertilizin mechanism on the process of fertilization has never been made clear. According to one of the dominating views, "fertilizin represents the specific receptor substance for the attachment of the sperm and is largely responsible for the tissue and species specificity of fertilization" [38]. Since fertilizin is believed to constitute the jelly layer of the eggs, one of the most important arguments in favor of these ideas has been the claim that sea urchin eggs deprived of their jelly layers should be more difficult to fertilize than normal eggs [26, 33, 34]. This argument has lost most of its importance since it has been shown that jelly free eggs of all sea urchin species thus far investigated, are easier to fertilize than normal eggs, provided the jelly layers are removed more carefully than in earlier work [3, 4, 5]. This new evidence does not, of course, eliminate the possibility that the principles exhibiting fertilizin activity may possess a

[1] The present discussion will be restricted to fertilization in the sea urchin.

physiological function, but it should be understood that this possible physiological function must be essentially different from that assigned to it formerly.

According to the data presented here, antibodies reacting with some predominating and specific chemical structure in the jelly layer of the *Paracentrotus* egg, only slightly (or not at all) inhibit fertilization, while antibodies reacting with the egg are strong inhibitors. Since the jelly layer of the *Paracentrotus* egg is known to possess a fertilizin activity ([26], p. 25), these results must either mean that the antibodies precipitating the jellies react with chemical structures unrelated to those exhibiting fertilizin activity, or that the number of sites saturated with antibodies under the conditions prevailing in the experiments is not sufficient to cause inhibition of the fertilization. It may mean, also, that the *improving* action on fertilization of the fertilizin principle in the jelly layer is slight or non-existent. In view of the evidence referred to above [3, 4, 5], the last-mentioned possibility seems most likely, but the point may be left open for discussion until more data have been collected.

According to the view of Tyler, fertilizin or "fertilizin-like substances" also constitute the sperm receptor of the egg surface [35, 36, 40, 41]. The meaning of the term fertilizin or "fertilizin-like substances" in this connection has never been very well defined. Are these receptors assumed to be structurally complementary to the same antifertilizin structures as the fertilizin principle of the jelly? Are they believed to react with different, "antifertilizin-like" structures of the sperm, or do they simply consist of molecules, chemically similar to the substance of the jelly layer and without any implications towards the fertilizin-active principles? Obviously, the term "fertilizin" is obsolete when applied in connection with the two last-mentioned possibilities. Probably there do exist substances in the cytoplasmic surface of the eggs, acting as specific receptors for the sperm, and these substances may very well be acid polysaccharides [11, 15, 30]; however, their active principles may be quite different from the fertilizin of the jelly layer. The evidence [35, 38, 39, 40, 41] given thus far in favor of a surface-fertilizin, structurally similar to the fertilizin of the jelly layer, is entirely indirect and not conclusive.

Tyler and Brookbank recently reported that antisera against fertilizin, when applied to fertilized and demembranated eggs, block cleavage much more strongly than do antisera against various extracts from sperm, eggs and embryos [38, 39]. The authors concluded that there are "surface constituents of the demembranated fertilized eggs that bear antigenic structures in common with fertilizin". This conclusion has apparently been

based on the assumption that "there is no macromolecular constituent other than fertilizin, constituting the gelatinous coat of the egg" [37]. Obviously, had there been several constituents, it would not have been possible for the authors to draw this conclusion. The evidence given for this assumption is (1) isolated jelly substances are electrophoretically and ultracentrifugally homogenous [37], and (2) their "purity can further be demonstrated by tests of absorption with homologous sperm" [38]. The last-mentioned evidence has been based on qualitative or semi-quantitative experiments [16, 37] without relevance to the problem concerned. Neither does the failure to detect heterogeneity in a substance of this type by means of physico-chemical methods prove that it is antigenically homogenous. These physico-chemical methods are not sufficient for detecting various high molecular impurities which occur in small amounts, and all of which may give rise to the production of antibodies [13]. But apart from that, there may occur an association of even larger amounts of chemically similar but antigenically different molecules—an association which may be difficult to resolve by any of the methods used. (For references and discussions cf. [12, 13, 31].) Thus, while the study of Tyler and Brookbank shows that antibodies against surface structures are effective cleavage-blocking agents, the conclusion that the findings constitute evidence for the presence of fertilizin or "fertilizin-like structures" in the egg surface remains to be substantiated. It may even be questioned whether the antigens responsible for the blocking antibodies were actually part of the jelly layer or part of the cytoplasmic egg surface. It has already been shown that injection of only small amounts of egg homogenates gives rise to antisera of very low activity when tested on the eggs [22]. The amounts of egg and embryo extracts injected by Tyler and Brookbank were, roughly, $\frac{1}{50}$ the amounts used in this study. Conversely, the amount of fertilizin injected by these authors was relatively large, and the substances were probably isolated from the eggs at pH 3–5 [36, 37]. By this procedure it is likely that substances are removed from the cytoplasmic egg surface. Antiserum prepared as described by the authors [38, 39] must be expected to exhibit various unrelated activities [22].

From the results presented in this paper it appears doubtful whether there exists, in the *Paracentrotus* egg, a surface-fertilizin similar to the jelly-fertilizin. The evidence given here suggests the occurrence, in the surface layers of the eggs, of a number of structurally highly different substances. Several of these substances may act as specific receptors for the attachment of the sperm. Evidence concerning the identity and chemistry of some of them will be presented in the following.

SUMMARY

Exposure of sea urchin eggs to antiserum induces a number of response reactions in the eggs. The following reactions, described previously, are further studied in this paper; (*a*) precipitation of the jelly layers of the unfertilized eggs, (*b*) parthenogenetic activation, (*c*) depression of the rate of fertilization, and (*d*) characteristic cortical damages in the fertilized eggs. Several variables governing the response of the eggs of the sea urchin *Paracentrotus lividus* are studied in detail. The relationship between the dose of antiserum and the response of the eggs is established. The findings constitute the basis for a comparative study of a number of different antisera. From the results of this study the following conclusions have been drawn.

1. The jelly layer of the eggs contains a particular antigen which gives rise to the formation of jelly precipitating antibodies when injected into rabbits. This antigen is located exclusively or mainly in the jelly layer.

2. One or several antigens are responsible for the formation of antibodies inducing the activation of the egg. These antigens belong to the egg rather than to the jelly layer but they may occur in the jelly layer as well. The jelly percipitating antibodies do not activate the egg.

The activating action of antiserum is enhanced when the egg-antiserum mixtures are transferred to the cold (4°C) immediately after mixing. Whereas this treatment favors cortical activation and the first steps of nuclear activation it suppresses cell division.

3. The inhibitory action of antiserum on the rate of fertilization is complex. It is due to the action of antibodies against egg antigens rather than against jelly layer antigens. The jelly precipitating antibodies are inactive and the antigens involved in this response do not seem to occur in the jelly layers.

4. One or several antigens are responsible for the formation of cortex-damaging antibodies. These antigens also belong to the eggs. They seem to be different both from the antigens involved in egg activation and from those involved in depression of fertilization rate. The jelly precipitating antibodies are inactive.

I wish to express my thanks to Professor John Runnström for his never-failing interest in my work and to the staff of the Stazione Zoologica for the generous supply of material and for their help during my stay at Naples.

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BRIEF NOTES

GASTRULAR BLOCKAGE OF FROGS' EGGS PRODUCED BY OXYGEN AT ATMOSPHERIC PRESSURE[1]

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Received June 24, 1957

Oxygen at partial pressures above one atmosphere prevents the gastrulation of *Rana pipiens* embryos. Nelsen [7] used 3.0 atmospheres of oxygen added to air at atmospheric pressure. The present author [4] more recently found 1.0 atmosphere of pure oxygen added to air to be effective. The partial pressure of oxygen was 1.2 atmospheres. Both workers begin oxygen treatment of the eggs shortly after fertilization and before the first cleavage. The dosage period ends when the embryos are in the later cleavage stages at which time they are transferred to medium in equilibrium with air at atmospheric pressure. These embryos are morphologically indistinguishable from control embryos raised since fertilization in air-equilibrated medium and develop to the middle or late blastula stage at the same rate [4, 5]. Depending on the dosage, however, gastrulation either proceeds abnormally or is inhibited completely, the embryos remaining in the late blastula stage for at least 30 hours before cytolyzing.

Since embryos develop normally when treated with 4.0 atmospheres of air [8] or 3.0 atmospheres of nitrogen added to 1.0 atmosphere of air, it was concluded that the effective factor in the treatment was oxygen and not the pressure, i.e., the mechanical force exerted per unit area [4]. In other words, oxygen is the toxic agent and damage to the embryos is a function of the concentration of oxygen in the 10 per cent Ringer's solution in which they are raised. This oxygen tension is in turn directly proportional to the partial pressure of oxygen in the gas phase above the culture medium (Dalton's Law) and varies inversely with the temperature [13]. The temperature at which 1.2 atmospheres of oxygen had been found to be effective in blocking gastrulation was 8.0°C. A drop of 3°C might still permit normal development of untreated embryos [6]. On the other hand, in the case of oxygen-treated embryos, it was thought that this temperature decrease with its concomitant increase in oxygen solubility might compensate for a decrease in oxygen solubility produced by reduction of the partial pressure of oxygen to 1.0 atmosphere, so that embryos raised in an oxygen-bubbled medium at 5°C would also be arrested at

[1] Supported by U.S. Public Health Service Grant No. A-1082 to John R. Gregg whose encouragement is gratefully acknowledged.

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TABLE I

Effect of exposure to 1 atmosphere of oxygen at 4.7° ± 0.05°C on gastrulation.

Female	Days of O_2 treatment	Per cent incompletely blocked gastrulae	Per cent completely blocked gastrulae	Total per cent abnormal gastrulae
S_2	11.1	0	100	100
	9.4	0	100	100
S_5	10.2	0	100	100
	9.0	0	100	100
S_6	9.0	1	99	100

gastrulation. Production of oxygen-poisoned gastrulae, using an oxygen tension not in excess of one atmosphere was desired in order to confirm the toxic action of high oxygen tension without recourse to high pressure. A second purpose was to avoid the use of cumbersome pressure apparatus in chemical studies of the mechanism of oxygen poisoning.

Eggs were obtained by artificial induction of ovulation and fertilized by routine procedures [10]. The eggs from each female were fertilized by sperm from a different male. Within one hour after fertilization at 12°–14°C, 250–300 eggs were placed in 250 ml Erlenmeyer flasks fitted with 2-hole stoppers, each with an inlet and outlet tube. Oxygen was bubbled through the medium (10 per cent Ringer's solution) for one hour after which both tubes were clamped off and the flasks transferred to a water bath at the desired temperature. At this point the embryos had not yet undergone cleavage. Controls were eggs from the same clutch exposed to an air-bubbling treatment, but otherwise handled throughout as were the oxygen-treated animals. After the desired duration of treatment, when the embryos were still in pre-gastrular stages, they were transferred to fresh medium (not oxygen-bubbled) at 9°C. Homogeneous samples of 100 normal blastulae were selected for future scoring for gastrular abnormality. This excluded any possible pre-gastrular abnormalities from the scoring which was performed when the control embryos were at the tailbud stage. The scoring was a census for the percentages of embryos falling into the following classes: (*a*) normal, (*b*) incompletely blocked gastrulae, consisting of abnormal gastrulae and neurulae (the abnormality in most of the latter was a persistent yolk plug), and (*c*) completely blocked (pre-)gastrulae, i.e., non-cytolyzing, very late blastulae appearing normal and corresponding to Shumway [12] stage 9 or 9+. Except where noted, less than 10 per cent of the control embryos were abnormal in each experiment. Until they appeared abnormal, oxygen-poisoned embryos developed at the same rate as did the controls.

The lowest temperature at which the embryos were reared during exposure to oxygen was 4.7° ± 0.05°C. The results from clutches of eggs from three females are recorded (seen) in Table I. At this low temperature oxygen had its full effect, *all* of

Table II

Effect of temperature on toxicity of 1 atmosphere of oxygen.

Temperature during O_2 treatment	Days of O_2 treatment	Per cent normal gastrulae	Per cent incompletely blocked gastrulae	Per cent completely blocked gastrulae	Total per cent abnormal gastrulae
9.5±0.1°C	4.9	97	3	0	3
7.2±1°C	6.8	0	99	1	100
4.7±0.05°C	9.0	0	1	99	100

the eggs in each sample being *completely* blocked at gastrulation. In the case of eggs from two more females, the same results were obtained, but the control embryos were abnormal; while in neither control sample were there any completely blocked embryos, there were 50 per cent incompletely blocked in one and 87 per cent in the other. At any rate, of the total of five females used for experiments at 4.7°C, control eggs from three survived the cold, while those from two did not fare so well, albeit better than those treated with oxygen.

When the same experiments were repeated at a slightly higher temperature (5.7° ±0.5°C) to avoid variability in the untreated embryos' tolerance to the cold, it was at the expense of the uniform and complete effectiveness of oxygen poisoning observed at 1.0°C lower. Control eggs from six of the seven females used were at least 95 per cent normal. While oxygen-treated eggs from only three of these six females showed a high percentage of completely blocked gastrulae (100, 100, 90 per cent), the total percentage of abnormal gastrulae (completely blocked plus incompletely blocked) was in every case 100 per cent.

A comparison of the results at 4.7°C and at 5.7°C indicated, as expected, that the toxicity of 100 per cent oxygen at atmospheric pressure was markedly dependent on the temperature. Furthermore, Ryan [11] has noted that frog embryos develop normally in an oxygen-bubbled medium at 18°C. To confirm the temperature-sensitivity of oxygen poisoning, samples of eggs from the same cross were kept at three different temperatures until the late blastula stage. The results are presented in Table II, showing the narrow temperature range within which 100 per cent oxygen has its effect. At 4.7° ±0.05°C, oxygen has its full effect; all the embryos are completely blocked. At a temperature 2.5°C higher (7.2° ±1°C) all the embryos are still abnormal, but are now incompletely blocked. With a further increase in temperature of 2.3°C, the oxygen has no effect, all the embryos gastrulating normally. Thus in order to affect gastrulation, oxygen at 1 atmosphere must be administered below 9.5° ±0.1°C.

In this series, a lower solubility of oxygen is undoubtedly not the only reason why the embryos develop normally at 9.5°C. The embryos develop faster at higher temperatures, permitting fewer hours of treatment before gastrulation begins, and this is another dosage variable [4, 5]. However, in the runs at 4.7°C and at 5.7°C, the lessened effect of oxygen at the higher temperature is attributable only to the

oxygen solubility, for the durations of treatment at 5.7°C were equal to or greater than those at 4.7°C. At 4.7°C as little as 9.0 days exposure to 1 atmosphere of oxygen produces 100 per cent completely blocked embryos (see Table I) and preliminary results indicate that 8.1 days treatment is equally effective.

Although as little as 1.0 atmosphere of oxygen prevents normal gastrulation of frog embryos if administered at 7.2°C or lower, showing conclusively that this is an oxygen effect and not a pressure effect, this phenomenon is not without parallel. Despite the fact that the great majority of oxygen poisoning effects are obtained with partial pressures of oxygen much above 1 atmosphere, Bean [1] does cite examples of the toxicity of oxygen tensions not in excess of 1 atmosphere, and recently Noell [9] has shown destruction of visual cells of the rabbit using oxygen at 55 per cent of ambient pressure.

Since the work of Paul Bert [2] in the late 19th century, hyper-atmospheric doses of oxygen have been known to be toxic, but now it is becoming apparent that even atmospheric doses can be poisonous, and that the "dose" is actually the concentration of oxygen in the tissue medium which is determined by the temperature as well as the partial pressure of the gas phase and the nature of the medium. Thus, when tissue or enzyme preparations "go bad," possibly this is due to oxygen poisoning, especially in those kept cold, despite the well-known preservative effect of cold under certain conditions on biological preparations. Inorganic phosphate, for example, induces swelling of mitochondria and uncouples oxidative phosphorylation. This may be an oxygen poisoning effect, for anaerobiosis affords protection, but CN^- does not, and the phosphate effects do occur with H_2O_2 under anaerobic conditions [3]. In addition, observations (to be published separately) of frog embryos raised in air at "lethal" temperatures (3°–4°C) indicate morphological effects similar to those of oxygen poisoning, i.e., specifically gastrular abnormalities [4, 5].

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THE NUCLEUS IN THE ACCUMULATION OF IRON BY LIVER CELL SUSPENSIONS

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Received June 25, 1957

In the course of our investigations concerning mechanisms for iron accumulation by rat liver slices [7, 8] it was found that iron, as ferric ammonium citrate, can be taken up against an apparent concentration gradient by a process which does not directly depend upon metabolic energy. Further, it was demonstrated by kinetic studies that the mechanism involves the sorption of the iron onto specific iron binding sites on or within the cell, possibly associated with ferritin [5]. To obtain a more intimate view of this process we have undertaken extensive investigations of iron uptake by suspensions of rat liver cells. The same basic chemical and physical properties of the iron accumulation system appear to be operative in both the liver slice and the cell suspension.

Rat liver cell suspensions were prepared by a modification of the method of Anderson [2]. Washed, loosely packed liver cells were suspended in cold Krebs-Ringer phosphate buffer, pH 7.0 (50 75 mg cells per ml buffer final concentration). Uptake experiments were carried out with shaking at 37°C in 15 ml conical centrifuge tubes. The reaction mixture contained 90 γ of iron (as ferric ammonium citrate) in 2.0 ml of Krebs-Ringer phosphate, pH 7.0, and 1.0 ml of cell suspension. The reaction was terminated at suitable time intervals by centrifugation for 1 minute at approximately 500 g, the supernatant drawn off, the cells washed once with 2.0 ml of cold, iron-free Krebs-Ringer, and the cells centrifuged again. The total amount of iron in each pellet was determined by the methods described in detail in a previous communication [7]. The results of a typical experiment to demonstrate the uptake of iron as a function of time are presented in Fig. 1. The shape of this curve is quite similiar to that observed in the experiments with liver slices [7]. However, less time is required by the cells to reach the saturation level. The order of magnitude of net accumulation is the same.

An attempt was made to localize the site of iron binding by cytological staining and microscopic observation. Smears were made of aliquots of liver cells incubating

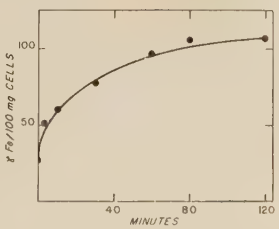
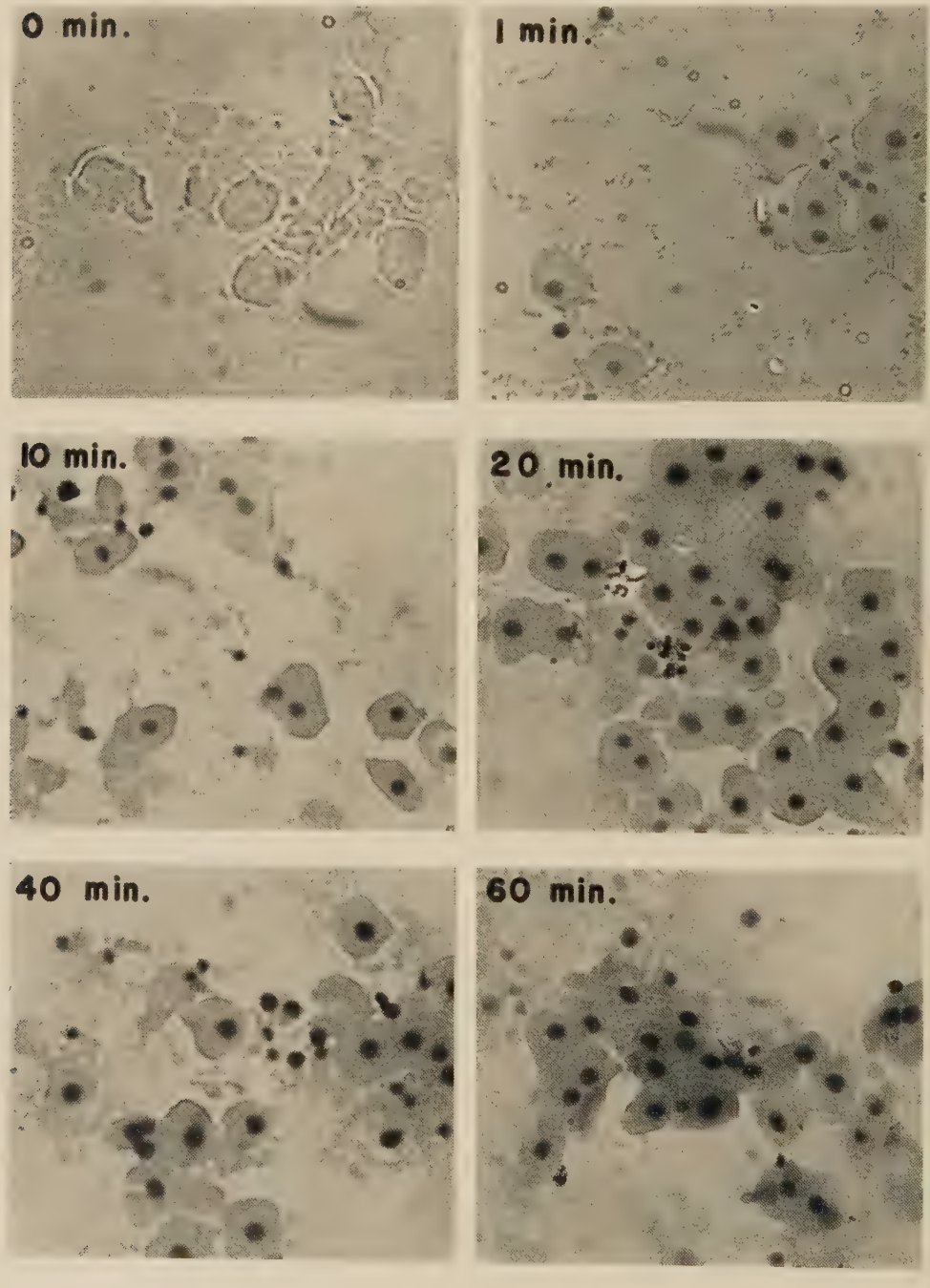


Fig. 1.—The accumulation of iron by a rat liver cell suspension as a function of time. The reaction mixture contains 90 γ of iron as ferric ammonium citrate in 2.0 ml Krebs-Ringer phosphate buffer pH 7.0, and 1.0 cell suspension containing 59 mg cells.


0 min.
1 min.
10 min.
20 min.
40 min.
60 min.

under the same conditions described above. These smears were fixed in formalin fumes and stained by Perl's ferrocyanide reaction [6] specific for iron. The photomicrographs of the stained cells at various time exposures to iron are shown in Fig. 2. The most striking aspect of this series is that the nucleus is the first cellular component in which iron is demonstrable. It appears that the nucleus is rapidly saturated with iron. The cytoplasm, on the other hand, accumulates the iron over a longer period. It is interesting that the iron present in the cell prior to exposure to exogenous iron (0 min.) is not visible with this technique.

It was necessary to determine whether or not the observations made in the experiments described above were, in fact, related to the biological uptake in the liver cells. Slices and cell suspension smears (not previously exposed to iron) fixed in formalin fumes were incubated for various periods of time with iron as indicated in the foregoing experimental procedures. When these tissues were stained, no detectable iron was observed. It seems likely, therefore, that the staining results reflect the mechanisms involved in the uptake of iron.

There is much direct evidence concerning the participation of various cellular components in the processes of ion accumulation, particularly the mitochondrion [4] and the cell membrane [5]. Recently, there has been some evidence that the nucleus may participate in the accumulation of various substances. Waddington [10] has implicated the nucleus in the incorporation of amino acids by avian and amphibian embryonic tissues. Experiments with bone marrow smears [1, 3] indicate that mature erythrocytes, which are anuclear, do not pick up radio-iron. The experiments presented here demonstrate that the nucleus plays a role in the uptake of iron. Studies are now in progress to determine whether iron accumulation can be carried out by isolated nuclear suspensions.

This work was supported by the United States Atomic Energy Commission and the Smith, Kline and French Foundation. We are grateful to the Allan Hancock Foundation for use of their facilities during this investigation.

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Fig. 2.—Photomicrographs of liver cells exposed to iron for varying periods, then stained by Perl's ferrocynanide reaction.

LOCALISATION DE L'ACIDE RIBONUCLÉIQUE DANS LES DIVERSES STRUCTURES MORPHOLOGIQUES DES MICROSOMES DE FOIE DE RAT

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Reçu le 9 juillet 1957

De récents travaux ont eu pour objet l'étude au microscope électronique des microsomes isolés par centrifugation différentielle et l'identification des différentes formations observées par rapport aux structures morphologiques existant dans la cellule *in toto* [1, 2, 3]. L'examen des coupes ultrafines de microsomes met en évidence, en particulier, (*a*) des formations vésiculaires, portant accolés à l'extérieur, des grains denses osmiophiles de 10 à 15 mμ de diamètre; elles correspondent à l'ergastoplasme de Dalton, d'Oberling et col., aux "rough surfaces" de Palade et Porter, et aux α-cytomembranes de Sjöstrand; (*b*) des vésicules dont la membrane est dépourvue de grains : ce sont les « smooth surfaces » de Palade.

Palade et Siekevitz pensent qu'au double élément structural des « rough surfaces » (membranes et grains) correspond une dualité de compositon : l'acide ribonucléique serait associé aux particules denses alors que la majorité des protéines et presque tous les phospholipides appartiendraient aux membranes ou au contenu des vésicules. Leur conclusion repose principalement sur les résultats obtenus en traitant les microsomes par le désoxycholate de sodium qui permet d'obtenir après centrifugation, un culot riche en acide ribonucléique (de 3 à 8 fois la concentration initiale) et composé presque uniquement de grains denses qui, selon ces auteurs, correspondraient aux particules osmiophiles de l'ergastoplasme observé sur les cellules *in toto* [3].

Nos propres résultats obtenus par le fractionnement des microsomes du foie de rat nous amènent à penser que l'acide ribonucléique n'est pas localisé uniquement dans les grains denses osmiophiles, mais qu'il en existe des quantités importantes dans les membranes des vésicules, ou dans leur contenu. Cette conclusion repose sur les faits expérimentaux suivants :

(1) Les microsomes isolés à partir d'un homogénéisat de foie à 10 % dans le saccharose 0,88 *M* ont, par mg d'azote protéique (NP), des teneurs en acide ribonucléique[1] et en phosphore lipidique[2] qui varient peu suivant les préparations. Sur 15 expériences, les valeurs trouvées sont respectivement 63 (déviation standard : 4,8) et 111 (déviation standard : 10,6). Nous n'avons jamais obtenu dans le domaine des microsomes de culots, soit pauvres en ARN, soit pauvres en PL. Ceci reste vrai si l'on fractionne les microsomes, quelle que soit la fraction envisagée.

(2) A cette constance relative de composition biochimique, s'oppose une grande variété d'aspects morphologiques. L'examen au microscope électronique des coupes ultrafines de culots de microsomes montre qu'à côté des vésicules ergastoplasmiques

[1] Exprimé en μg de phosphore ribonucléique : P.ARN.
[2] Exprimé en μg de phosphore : PL.

typiques, bordées de grains (les « rough surfaces » de Palade), on décèle la présence de vésicules plus petites, non bordées de grains (les « smooth surfaces »). Palade et Siekevitz notent la prédominance des « rough surfaces » dans leurs préparations (85 %).

Or ce qui nous a frappé, dans les nombreuses préparations de microsomes que nous avons faites à partir de foies prélevés sur des rats Wistar normaux, c'est la grande variabilité de cette proportion.

Après fractionnement des microsomes totaux, certains culots sont presque uniquement composés de formations membraneuses munies de grains; par contre, d'autres sont constitués d'une majorité de vésicules de tailles variables, sans grains. Il peut exister aussi, dans certains cas, une grande quantité de grains libres. Quelle que soit la proportion relative de ces trois composants morphologiques, les analyses biochimiques montrent que les rapports P.ARN/NP et PL/NP rentrent tous dans la marge de variations indiquées plus haut; il n'y a donc pas de relation directe entre la concentration d'un culot en acide ribonucléique et la proportion de grains, fixés sur les membranes; en particulier, le fait de travailler sur un matériel privé de « rough surfaces » n'a pas pour conséquence, une chute parallèle du rapport P.ARN/NP.

Par contre, dès que l'on s'adresse à des culots postmicrosomiaux (obtenus en centrifugeant le surnageant des microsomes), on constate une chute brutale de la teneur en acide ribonucléique et phospholipides par mg d'azote lorsque les éléments structurés disparaissent au profit des macromolécules protéiques.

(3) Nous avons fait agir le désoxycholate de sodium dans des conditions voisines de celles de Palade et de Siekevitz [3] sur plusieurs fractions de microsomes qui se distinguaient par leur composition morphologique. Deux étaient très riches en ergastoplasme et même en grains libres et avaient comme rapport : P.ARN/NP = 55,7 et 73,6; les deux autres ne contenaient que très peu de formations ergastoplasmiques, mais une grande quantité de « smooth surfaces » et avaient comme rapport : P.ARN/NP = 64 et 63. Après traitement au désoxycholate et centrifugation de 4 h. à 150.000 *g* les culots finaux présentent les caractéristiques suivantes :

(*a*) au point de vue morphologique, ils sont tous constitués de particules denses granulaires,

(*b*) leur composition biochimique est P.ARN/NP = 296; 254; 208 et 239 et PL/NP = 1,8; 1,8; 1,8 et 2,3,

(*c*) ils représentent respectivement 54; 53; 51,6 et 40 % de l'acide ribonucléique initial de 10; 15; 16 et 10,5 % de l'azote protéique.

Cet ensemble de résultats montre que l'acide ribonucléique des microsomes du foie n'est pas localisé uniquement sur les grains de formations ergastoplasmiques ou « rough surfaces », mais qu'il en existe des quantités importantes dans les membranes des vésicules, ou dans leur contenu. Ceci permet de comprendre que, quelle que soit la proportion des différents composants morphologiques, les microsomes ont toujours une teneur en ARN sensiblement constante. Il nous semble d'un grand intérêt théorique de constater que le désoxycholate de sodium libère non seulement les grains denses, visibles sur les coupes, mais qu'il provoque à partir des membranes, la « libération » de particules ayant une constitution chimique analogue; on peut dire que ces formations riches en ARN préexistaient sous une forme morphologiquement indistinguible en raison de liaisons différentes entre les divers constituants de la membrane.

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THE EFFECT OF SULFHYDRYL INHIBITORS AND THIOL COMPOUNDS ON PIGMENT AGGREGATION AND DISPERSION IN THE MELANOPHORES OF *ANOLIS CAROLINENSIS*[1]

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Received July 11, 1957

Sulfhydryl inhibition causes the dispersion of the melanin granules in the melanophores of the frog skin [4] and to this extent simulates the action of the melanocyte stimulating hormone (MSH). However, treatment of the frog skin with the thiols diethyldithiocarbamate, cysteine and glutathione are reported to have no effect on the action of the hormone [5]. In a recent report [3] a preparation of the skin of the lizard *Anolis carolinensis* was described which, by virtue of its magnitude of response, homogeneous appearance and particularly its permeability, seems better suited for investigations into melanophore physiology and MSH action than the frog skin. It is the intent of this communication to present findings on the effect of thiol compounds and sulfhydryl inhibitors on *Anolis* skin melanophores suggestive of a substantial involvement of sulfhydryl groups with MSH action.

It was found in the lizard skin preparation that the SH inhibitors NEM[2] and

[1] This investigation was supported in part by grants from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and by the United States Public Health Service Grant #RG-2912 (C4). The work was done while a John M. Prather Research Fellow of the University of Chicago and a National Science Foundation Predoctoral Fellow in the Department of Zoology of the University of Chicago.

The Author wishes to express appreciation for the advice and assistance of Dr. Dorothy Price and Dr. H. G. Williams-Ashman.

[2] The agents used and their abbreviations are as follows: N-ethyl maleimide (NEM), adenosine triphosphate (sodium salt) (ATP) reduced glutathione and cysteine were obtained from the Nutritional Biochemical Corporation. Mersalyl (Salyrgan), the sodium salt of O-[(3-hydroxymercuri-2-methoxypropyl) carbamyl] phenoxyacetic acid was a gift of Dr. E. S. Guzman-Barron. The pituitary extract used in this study was made available by Dr. Irby Bunding, Armour Laboratories, Chicago. It is lot #D216-155C and is an extract of hog pituitary. Its MSH potency is, roughly, 1.4×10^3 units/g as determined by Drs. Shizume and Lerner, according to their method [5]. The Ringer solution used in these experiments was the same as previously described [3].

mersalyl have a complex effect on melanophores. The results with NEM are given in Fig. 1. Titers of 0.1 and 1.0 mM of NEM give complete dispersion (Fig. 1*b* and 1*c*). At 10.0 mM of NEM full dispersion is not achieved (Fig. 1*a*). The action of NEM is inhibited by pretreating the skin with cysteine (Fig. 1*e*) or glutathione (Fig. 1*d*).

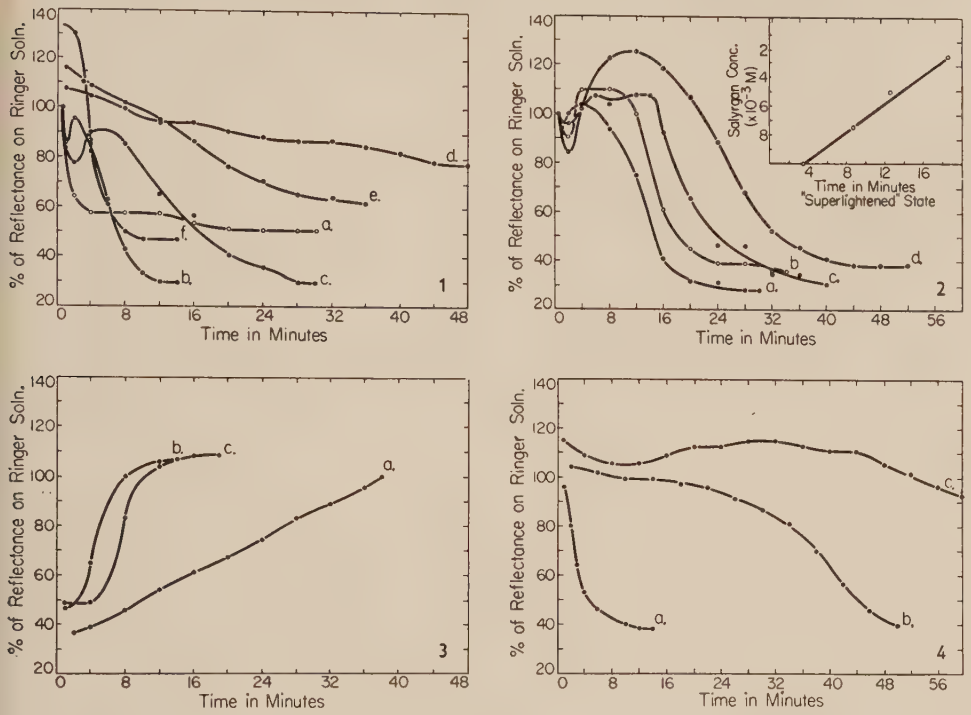

Fig. 1.—The dispersion of pigment within the melanophores of *Anolis carolinensis* skin floated on N-ethylmaleimide solution. *a*, Skin floated on 10^{-2} M N-ethylmaleimide. *b*, Skin floated on 10^{-3} M N-ethylmaleimide. *c*, Skin floated on 10^{-4} M N-ethylmaleimide. *d*, Skin floated on 10^{-3} M N-ethylmaleimide following treatment with 10^{-3} M reduced glutathione for 20 min. *e*, Skin floated on 10^{-3} M N-ethylmaleimide following treatment with 10^{-3} M cysteine for 20 min. *f*, Skin floated on 10^{-3} M N-ethylmaleimide following treatment with 10^{-3} M ATP (di Na salt) for 20 min.

Fig. 2.—The dispersion of pigment within the melanophores of *Anolis carolinensis* skin floated on mersalyl solutions. *a*, Skin floated on 10^{-2} M mersalyl. *b*, Skin floated on 7.5×10^{-3} M mersalyl. *c*, Skin floated on 5.0×10^{-3} M mersalyl. *d*, Skin floated on 2.5×10^{-3} M mersalyl. *Insert:* The length of time skin treated with mersalyl (Salyrgan) remains in the "superlightened" condition plotted as a function of mersalyl concentration.

Fig. 3.—The dispersion of pigment within the melanophores of *Anolis carolinensis* skin floated on MSH. *a*, Skin floated on Ringer solution containing 1.4×10^{-7} g/ml of MSH rich pituitary extract. *b*, Skin floated on 1.4×10^{-7} g/ml pituitary extract following treatment with 10^{-3} M reduced gluthathione for 20 min. *c*, Skin floated on 1.4×10^{-7} g/ml pituitary extract following treatment with 10^{-3} M cysteine for 20 min.

Fig. 4.—The aggregation of pigment within the melanophores of *Anolis carolinensis* skin. *a*, Skin floated on Ringer solution following treatment with 1.4×10^{-7} g/ml MSH rich pituitary extract for 10 min. *b*, Skin floated on 10^{-3} M reduced glutathione following treatment with 1.4×10^{-7} g/ml pituitary extract for 10 min. *c*, Skin floated on 10^{-3} M cysteine following treatment with 1.4×10^{-7} g/ml pituitary extract for 10 min.

Though ATP has been shown to inhibit the darkening action of MSH [2, 3] pretreatment of the skin with ATP does not materially effect the rate of darkening on NEM, though it results in slightly incomplete darkening (Fig. 1*f*).

Besides differing in the degree of darkening achieved on 0.1 and 1.0 m*M* NEM as compared to 10.0 m*M* NEM another difference may be noted. Skin darkened at the lower titer of NEM does not darken evenly but darkens rapidly for about a minute then lightens slightly and darkens finally to full darkness (Fig. 1*b* and 1*c*). This temporary reversal of the darkening trend is a consistent feature of darkening on low titers of NEM.

A clearer picture of the complex effect of the action of SH inhibitors is obtained by using mersalyl. This agent is of greater molecular weight than NEM and appears to diffuse into the cell more slowly.

Fig. 2 shows that if the temporary reversal seen in darkening in low titers of NEM is induced by mersalyl it is greatly augmented. At the height of the reversal the skin is always lighter than it was on Ringer solution (i.e. in the absence of hormonal influence). The length of time required for the reversal to occur, is, within the range of concentration tested, independent of concentration. The inset in Fig. 2 shows that the length of time the skin remains in the superlightened condition is an inverse function of the inhibitor concentration, that is the length of time required for the final darkening action of mersalyl to dominate the lightening action is inversely proportional to concentration.

Once final darkening has taken place with NEM or mersalyl it is not reversible with Ringer solution, glutathione or cysteine.

The complex effect of SH inhibitors on melanin dispersion and aggregation suggested the possibility that compounds capable of protecting SH groups may have some effect on MSH action. This suspicion has been confirmed. Pretreatment of green skin with glutathione (Fig. 3*b*) or cysteine (Fig. 3*c*) leads to marked inhibition of darkening when the skin is placed on MSH. Pretreatment of skin with ascorbic acid does not effect the action of the hormone.

If skin is darkened on MSH its rate of lightening upon removal of the hormone is markedly increased by the presence of glutathione (Fig. 4*b*) or cysteine (Fig. 4*c*). Lightening on these agents terminates in a slightly superlightened condition.

Dixon [1] found that MSH may be inactivated by treatment with hydrogen peroxide. This inhibition cannot be released with potassium borohydride but can be reversed with thioglycollate. It is therefore unlikely that the action of thiols in the present experiments is on the hormone itself. The alternative hypothesis is that thiol compounds act on the melanophore and/or the MSH-melanophore bond in some way which prevents the action of MSH. The observation that sulfhydryl inhibitors mimic MSH in causing darkening suggest that MSH may be acting to oxidize SH groups. This is not inconsistent with the evidence presently available.

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TENEUR EN ACIDE DÉSOXYRIBONUCLÉIQUE ET VOLUME DES NOYAUX DES CELLULES HÉPATIQUES CHEZ L'HOMME

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Reçu le 11 juillet 1957

De nombreux auteurs ont déterminé par des méthodes histophotométriques la teneur en DNA des noyaux des cellules hépatiques chez le rat et la souris. Ils y ont constamment trouvé trois classes nucléaires dont les teneurs moyennes se rapportent comme les termes d'une progression géométrique. Il est connu depuis longtemps déjà que ces cellules présentent également des classes nucléaires volumétriques, et il a été montré [3] que classes volumétriques et classes de teneur en DNA se superposent. Ces classes nucléaires sont vraisemblablement le fait d'un phénomène d'endopolyploïdie, les cellules correspondantes étant diploïdes, tétraploïdes et octoploïdes.

On en est imperceptiblement arrivé bien souvent à généraliser ces données et à considérer le foie en général conme un organe à classes nucléaires. Or, Fautrez et Moerman [4] ont pu montrer chez le téléostéen *Lebistes reticulatus* la présence d'une seule classe nucléaire (diploïde) dans les cellules hépatiques. Bien plus, dans une espèce aussi voisine du rat et de la souris qu'est le cobaye, Daskalides [1] ne trouva également qu'une seule classe nucléaire dans le foie, et ceci tant en ce qui concerne

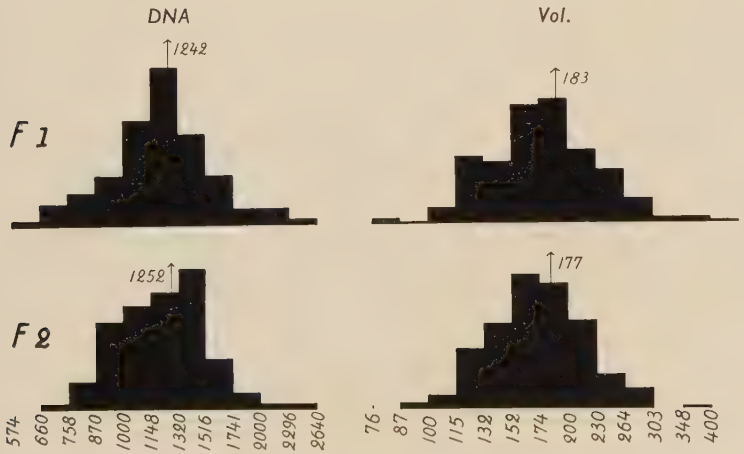

Fig. 1.—Histogrammes concernant la teneur en DNA (en unités arbitraires) et le volume (en μ^3) des noyaux des cellules dans deux foies humains.

[1] Travail réalisé grâce à un subside du Fonds National belge de la Recherche Scientifique.

la teneur en DNA, qu'en ce qui concerne le volume. Il n'était dès lors pas dépourvu d'intérêt de voir comment se comporte le foie humain à ce point de vue.

Nous avons pu disposer de deux fragments de foie humain, provenant de deux individus différents, prélevés par biopsie et fixés dans d'excellentes conditions dans de l'alcool-formol acétique (80 parties d'alcool 96°, 15 parties de formol, 5 parties d'acide acétique glacial). L'examen anatomo-pathologique n'ayant révélé aucune

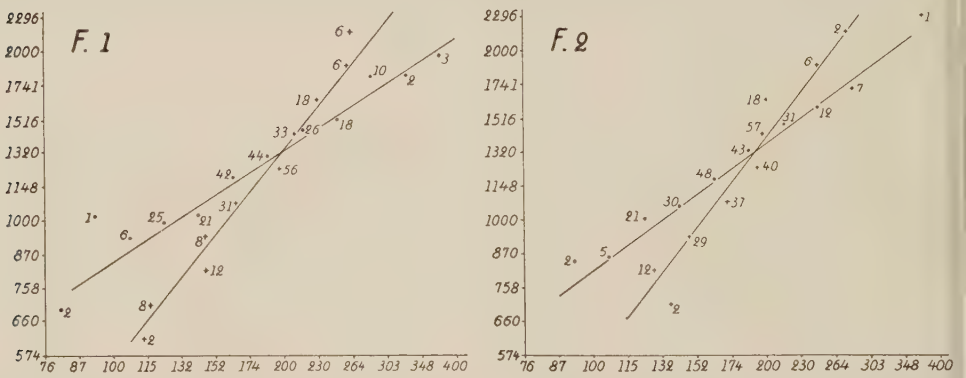

Fig. 2.—Lignes de régression DNA/Volume et Volume/DNA dans les noyaux de deux foies humains.

altération pathologique, des coupes de 10 μ des 2 fragments furent portées sur une même lame. La coloration au Feulgen fut donc rigoureusement identique dans toutes les pièces examinées. La teneur en DNA fut déterminée dans les noyaux *in situ* par la méthode histophotométrique de Lison [5]. Les noyaux étant pratiquement sphériques, leur volume (en μ^3) fut déduit par une formule géométrique simple de leur surface de projection, en tenant compte du grossissement.

La fig. 1 rassemble les histogrammes qui résument nos résultats; leurs caractéristiques statistiques sont indiquées dans le tableau I.

Les résultats obtenus sur les biopsies provenant du foie de deux individus différents sont remarquablement superposables. On voit que dans le foie humain il n'existe, tant en ce qui concerne le volume nucléaire qu'en ce qui concerne la teneur en DNA, qu'une seule classe de noyaux. La teneur moyenne en DNA est de l'ordre de celle que nous avons constamment retrouvée dans des conditions expérimentales semblables, dans des cellules diploïdes de mammifères.

Tableau I

	Teneur en DNA en unités arbitraires			Volume en μ^3		
	$M \pm s\bar{x}$	S	n	$M \pm s\bar{x}$	S	n
Foie I	1242 ± 23	329	200	183 ± 3,7	52	200
Foie II	1252 ± 21	300	200	177 ± 3,0	85	200

Nous avons enfin voulu nous rendre compte si dans cette classe nucléaire il existe pour les divers noyaux individuels une corrélation entre le volume et la teneur en DNA. Les lignes de régression DNA/Volume et Volume/DNA (fig. 2) ainsi que la valeur du coëfficient de corrélation *r*, égale à 0,448 pour le foie I et à 0,680 pour le foie II montrent que cette corrélation est réelle. Ces résultats sont semblables à ceux que l'auteur senior [2] obtînt dans les organes de rat à une seule classe nucléaire.

Résumé — conclusions. Le foie humain ne contient qu'une seule classe nucléaire. Il s'agit très vraisemblablement de noyaux diploïdes. Au sein de cette classe la corrélation entre le volume et la teneur en DNA des noyaux individuels est réelle.

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CYTOLOGICAL DEMONSTRATION OF SEX IN FRESH UNSTAINED PREPARATIONS UNDER THE PHASE CONTRAST MICROSCOPE

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Received July 15, 1957

The resting nuclei of normal females contain a special chromatin mass, the sex chromatin, which is seldom seen in nuclei of normal males. This special structure was first seen by Barr and Bertram [1] in the nerve cell of the cat, but since then it has been shown that similar structures are present in somatic nuclei in various species of animals and in different tissues. Moore and Barr [3, 4] demonstrated its occurrence in human tissues.

Since the sex chromatin is Feulgen-positive, indicating the presence of DNA, the available evidence suggests that sex chromatin is derived from the sex chromosomes. It is probable that heterochromatic portions of the XX chromosomes of female nuclei form a mass which is sufficiently large to be readily identified. In the male, the relative small size of the Y chromosome even when coupled with the X component, renders the mass inconspicuous. Whatever the actual explanation may be, the presence or absence of the so-called sex chromatin helps in identifying the genetic sex of the individual.

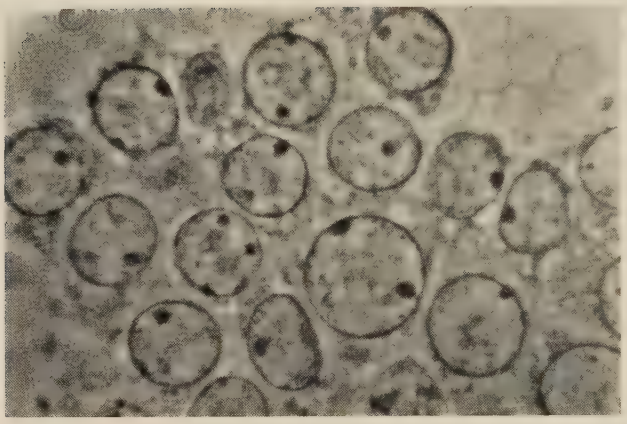

Fig. 1.—Human cell nuclei showing sex chromatin in fresh unfixed unstained preparations as seen under the phase contrast microscope. × 1200.

Cytological investigation of sex can be made in fresh unfixed and unstained material under the phase contrast microscope. The material obtained is mixed on a glass slide with normal saline, covered with a coverglass and observed with oil immersion lenses. Observation with a magnification above 900 diameters is necessary in order to visualise sex chromatin.

Sex chromatin usually appears as a dense, semi-spheroid or plano-convex mass, ½–2 microns in diameter, adhering to the inner surface of the nuclear membrane. Occasionally it may be triangular in shape, the free vertex facing the nucleoplasm. The sex chromatin can rarely be confused with a nucleolus since the latter is larger, spherical in shape, and usually located at some distance from the nuclear membrane. It is only with great rarity that two structures with the size and location of sex chromatin are encountered in the same nucleus.

In order to ascertain the proportion of sex chromatin contained in cell nuclei of a given material we have studied 200 to 400 cells from different fields and deduced the average percentage. Since the most typical location is peripheral, our counts are based on this appearance.

In 300 morphologically normal individuals whose sex we have investigated in scrapings from the oral, vaginal or urethral mucosae we have found from 1 to 20 per cent sex chromatin in the nuclei of resting cells in males and from 24 to 65 per cent in those of females.

When there is a great number of bacteria in the material, interpretation is very difficult. Not only can the sex of cells be demonstrated in fresh preparations, but also their cytological characteristics, especially malignancy, can be simultaneously detected [2].

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THE FINE STRUCTURE OF NATIVE COLLAGEN IN THIN SECTIONS[1]

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Received July 20, 1957

Our knowledge of the fine structure of collagen, as far as it can be demonstrated with the electron microscope, has been derived almost exclusively from observations on isolated microfibrils. Only recently have ultrathin sections been used successfully to study the intraperiodic banding of the collagenous microfibrils, by Rhodin and Dalhamn [4]. However, these authors were unable to resolve the periodic structure of the fibrils to the degree accomplished by users of whole mounts, such as Gross and Schmitt [1], who have demonstrated six intraperiodic bands, and Nemetscheck, Grassmann, and Hofmann [3], who distinguished up to 13 subdivisions in the 640 Å period. In view of the still existing uncertainties, new observations about the structure of collagenous microfibrils in ultrathin sections seemed to be desirable.

Materials and Methods.—Occasional observations in sections suggested the *lamina propria* of the mucous membranes of the pharynx in vertebrates as a tissue well suited for our purpose. Following ether anesthesia, small portions of pharynx of an adult male frog (*Rana pipiens*) were excised and fixed in 1 per cent osmium tetroxide in 0.72 per cent sodium chloride and citric acid-phosphate buffer (pH 7.4, 0.05 *M*) for 1 hour at room temperature. Following this, the tissues were transferred to 6 per cent formalin in 0.72 per cent sodium chloride for fifteen minutes and dehydrated by single, twenty-minute changes of 50 per cent, 75 per cent, 95 per cent, and absolute ethyl alcohols. After three 30-minute infiltration changes, the tissues were embedded in a mixture of 60 per cent *n*-butyl methacrylate, 40 per cent ethyl methacrylate, and 1 per cent luperco CDB by ultraviolet irradiation at 28°C.

The sections were cut on a Porter-Blum microtome set at one-fortieth micron and mounted on pure carbon membranes. The microscope was the RCA EMU-3A operated with a 100-Kv beam and a one-mil objective aperture. Micrographs were taken at original magnifications of 12,000 diameters or less on Kodak medium lantern-slide plates and printed on Kodabromide F 3 to F 4 paper. Since our sections were not thicker than $\frac{1}{40}$ μ or 250 Å, and the diameter of the microfibrils is of the order of 425 to 650 Å, practically all the fibrils shown in our pictures must be sectioned.

Observations. -The microfibrils are packed into bundles apparently consisting of several hundred, all of which show a periodic structure if sectioned in the longitudinal axis (Fig. 1). The diameter of the microfibrils range from 425 to 600 Å as measured in the sections, and they give no indication either in cross-section or oblique sections of having a tubular structure as reported by Kennedy [2]. In all longitudinal sections, the intraperiodic bands completely traverse the microfibrils and therefore appear to be discs spreading completely across. The periodicity averages 534 Å and ranges from 500 to 565 Å.

Six intraperiodic bands have been observed in periods cut at optimal thickness

[1] This work was performed under the auspices of the U.S. Atomic Energy Commission.

(Fig. 2). Measurements may show some variation in intraperiod spacings but in the typical case, the following are observed. The six bands have thicknesses of 50, 30, 30, 45, 45, and 35 Å, and the six spacings (beginning with space between the first two bands above) are 50, 30, 70, 50, 50, and 50 Å. (The above measurements cannot be considered rigidly indicative of all observed periods but will serve to emphasize certain of the more salient structural features.) The second and third bands are quite narrow and are placed close together. The space between the third and fourth bands is larger than other intraband spacings. The last band is quite thin and difficult to visualize.

Occasionally, the thicker bands are apparently resolved into granules. The first band especially has been resolved into five or six granules in a straight row as seen in section. These granules are about 50 Å in diameter with distances of separation of a similar dimension. The orientation is that of granules in a single plane, perpendicular to the long axis of the microfibril. These elements have been noticed by Gross and Schmitt, and their possible singificance was recently stressed by Wassermann [5]. Longitudinal connections between the granules, as described by Wassermann, can be found at some places in our pictures.

Neighbouring fibrils, even if not in contact, are in phase as to their periods and also as to the intraperiodic bands. Longitudinally cut fibrils show no demarcation that suggests an interface. The surface of cross-sections, however, is sometimes marked by a line which is slightly darker than the rest of their substance (arrow, Fig. 1). The cross-sections are evenly filled by a material which is darker than that outside the fibrils.

The cross-sectional view of the bundle of microfibrils in the right upper corner of Fig. 1 shows the space between the single fibrils to be of somewhat greater density than that of the background outside the bundle. This may indicate the presence of a material corresponding to the cement substance of the microfibrils which has been demonstrated by Wassermann [5] between incompletely separated fibrils.

Discussion.—Gross and Schmitt [1] have proposed the terminology which denotes the intraperiodic bands as *a*, *b*, *c*, *d*, and *e* and have shown that the *b* band is composed of two components designated b_1 and b_2. The dimensions given above are in the order of this scheme so that the six bands observed here coincide with the *a*, b_1, b_2, *c*, *d*, and *e*. The observations of Gross and Schmitt were made on isolated microfibrils both from fragmented frozen sections and from air-dried specimens and with both phosphotungstic acid (PTA) staining and shadowcasting techniques. PTA staining allowed the resolution of six bands but as many as five could also be resolved by chromium shadowing.

Rhodin and Dalhamn [4], who were the first to show the intraperiodic bands in osmium-fixed, sectioned collagen microfibrils from the *lamina propria* of the tracheal mucosa of the rat, have demonstrated the *a*, *b*, *c*, and *d* bands but have failed to resolve the double nature of the *b* band and have not demonstrated the *e* band. They report that the *b* band has the same width as the others, but our observations are not in agreement; in the frog, the combined b_1 and b_2 bands would produce a single band of greater width than any of the others. The difference is perhaps accounted for by a slight variation in structure due to species difference or other variations.

Nemetscheck, Grassmann and Hofmann [3] have demonstrated as many as thirteen intraperiodic bands in microfibrils isolated from the human ligamentum nuchae.

Fig. 1.—Survey micrograph of a bundle of microfibrils from the *lamina propria* of the pharynx of the frog, *Rana pipiens*. The microfibrils are shown to be "in-phase" when in contact with neighboring fibrils. No line of interface demarcation or membrane is visible at the edges of the microfibrils except in a few cross-sectional views (arrows). ×57,000.
Fig. 2.—A single, longitudinally sectioned microfibril showing the intraperiod bands. A set of six lines has been placed on the micrograph indicating the *a*, b_1, b_2, *c*, *d*, and *e* bands with the *a* band uppermost. ×85,000.

Most of these additional bands can be correlated with the system of Gross and Schmitt and can be recognized as further sub-bands rather than as new bands, so that the *a*-band, for example, is divided into three sub-bands. The thirteen bands, however, were not considered to represent the norm. Nemetscheck, Grassmann and Hofmann believe that a high degree of subdivision depends on the pre-treatment of the material and also on the amount of heat to which sections are exposed during observation under the electron microscope. The number of sub-bands may be therefore a variable structural phenomenon. As has been shown here, the larger of the existing bands can be resolved into granules, and, therefore, may be looked upon not as homogeneous sheaths or plates but rather as a plane of oriented granules similar to a crystalline lattice plane. It is conceivable that these agglomerations of material, which appear as granules, may be of such a nature as to be susceptible to separation into two or three granules. If the change concerns all the granules in one plane two or more sub-bands may result. This suggestion was derived from certain observations by the senior author ([5], p. 277). The experiments of Nemetscheck, Grassmann and Hofmann concerning the effects of heat and X-rays on the structure of the microfibrils seem to support this concept.

Thus, the findings of Nemetscheck, Grassmann and Hofmann do not contradict those of Gross and Schmitt, nor our own. The agreement between the observations made on isolated and on sectioned microfibrils proves that the techniques of embedding and sectioning are reliable in preserving the collagenous structures.

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OsO_4-FIXIERUNG INTRAZELLULÄRER MYELINFIGUREN[1]

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Eingegangen am 24. Juli 1957

In der Milz von Kaninchen, die einen gesteigerten Abbau von Erythrocyten und Leukozyten zeigten [21], beobachteten wir im Cytoplasma von Makrophagen häufig Strukturen, die wir als Myelinfiguren deuten. Dabei wurde die folgende Technik verwendet: Fixierung in gepufferter isotonischer OsO_4-Lösung nach Rhodin, Einbettung in Methacrylat über Alkohol und Aceton und Herstellung von Dünnschnitten nach dem in der Elektronenmikroskopie üblichen Verfahren mit einem Mikrotom nach Huppertz und v. Borries. Aufnahmen mit einem ÜM 100 e der Firma Siemens.

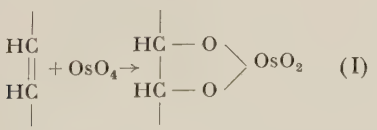
[1] Durchgeführt mit Unterstützung der Deutschen Forschungsgemeinschaft.

Myelinfiguren entstehen aus Lipoiden durch Aufnahme von Wasser. Es handelt sich meist um Kugeln oder Zylinder, deren Wand aus konzentrischen bimolekularen Lipoidlamellen besteht, zwischen die Wasserschichten eingelagert sind. Innerhalb einer Lamelle liegen die langen Fettsäureketten der Lipoidmoleküle parallel nebeneinander und einander zugekehrt. Die hydrophilen Gruppen zeigen nach aussen [15, 19, 13, 9].

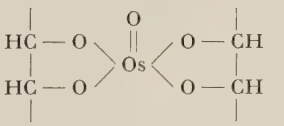
Bei den von uns beobachteten Myelinfiguren handelt es sich um Anschnitte von Röhren oder Hohlkugeln mit einer sehr elektronendichten Wand. Bei guter Auflösung lässt sich in der Wand eine konzentrische Schichtung aus abwechselnd hellen und dunklen Streifen nachweisen (Abb. 1). Der Abstand von der Mitte eines dunklen Streifens bis zur Mitte des nächsten dunklen beträgt 45–50 Å, die Dicke eines dunklen Streifens 25–30 Å, die des hellen ist etwas geringer. Es finden sich aber gelegentlich auch etwas lockerer gebaute Figuren mit breiteren hellen Streifen bei gleichbleibender Dicke der dunklen. Häufig liegen diese Strukturen am Rand — seltener in — einer grösseren homogenen elektronendichten Masse, in der auch einzelne Systeme annähernd paralleler Lamellen enthalten sind, die nicht die Anordnung in Ringen zeigen. Man hat den Eindruck, dass die Myelinfiguren aus dieser Masse entstehen.

Folgende Gründe führten zur Deutung dieser Strukturen als Myelinfiguren. Geren u. Schmitt [11] haben an Myelinfiguren aus reinen Acetalphosphatiden durch den Vergleich elektronenmikroskopischer Schnittbilder mit dem Röntgendiagramm gezeigt, dass die Struktur der Myelinfiguren nach OsO_4-Fixierung im elektronenoptischen Dünnschnitt erhalten bleibt und die in beiden Fällen gemessene Periode gleich gross ist. Die Entstehung von Myelinfiguren beim Zerfall von Erythrozyten *in vitro* ist mehrfach beschrieben [10], ältere Lit. bei [18]. Phosphatide, besonders Kephalin und freies Cholesterin sind die in grösster und etwa äquimolekularer Menge vorhandenen Lipoide des Erythrozyten [17]. Von ihnen neigt vor allen Dingen Kephalin zur Bildung von Myelinfiguren und teilt diese Fähigkeit auch anderen Lipoiden mit, die sie allein nicht besitzen [13, 19, 3, 16]. Finean [8] hat auf Grund von Röntgendiagrammen eine Vorstellung entwickelt, wie Cholesterin und Phosphatide zusammen eine bimolekulare Lamelle bilden, deren Dicke 50 Å beträgt. Das stimmt gut mit der von uns im Elektronenmikroskop beobachteten Periode überein. Daher können die einzelnen Lipoidlamellen unserer Myelinfiguren kaum durch breitere Zwischenräume getrennt sein. Die abwechselnd helle und dunkle Streifung im elektronenoptischen Bild muss demnach auf einer ungleichmässigen Verteilung des zur Fixierung verwandten Osmiums innerhalb der Lamelle beruhen, da der Kontrast nach Bahr [2] im wesentlichen durch die Osmiumverteilung bestimmt wird.

Gewöhnlich wird, gestützt auf die Arbeiten von Criegee [5, 6] eine Anlagerung des OsO_4 an die Doppelbindung der ungesättigten Fettsäurereste der Lipoide angenommen (I).



(I)



(II)

Diese für verschiedene Olefine nachgewiesene Verbindung entsteht beim Arbeiten in organischen Lösungsmitteln und ist wenig stabil. In wässriger Lösung entsteht

dagegen, wie Criegee in derselben Arbeit zeigt, eine andere unlösliche, sehr stabile Verbindung mit zwei Olefinmolekülen, die als Diester der hypothetischen Osmiumsäure H_4OsO_5 aufgefasst werden kann (II).

Eine solche Vernetzung benachbarter Fettsäureketten in den Lipoidlamellen würde die Stabilisierung der Myelinfiguren durch die OsO_4-Fixierung sehr gut erklären. Man müsste dann eine Anordnung der Doppelbindungen der Fettsäurereste in Ebenen senkrecht zum Radius der Myelinfiguren annehmen, was Finean [7] bereits aus dem Röntgendiagramm gefolgert hat. Da auch aromatische Doppelbindungen mit OsO_4 reagieren [1, 5, 6], erscheint auch eine Verknüpfung über die Doppelbindung des Cholesterins möglich.

Der Diester (II) entsteht nach Criegee aus dem „Monoester" (I) durch hydrolytische Spaltung und nachfolgende Reaktion des so entstandenen Diols mit noch vorhandenem Monoester oder auch aus zwei Molekülen Diol und Osmiumsäure. Die vorhergehende Oxidation der Doppelbindungen zu Diolen würde zu einer Ablagerung niedrigerer Oxidationsstufen des Osmiums führen und die auftretende Schwärzung erklären sowie zur Steigerung des elektronenoptischen Kontrastes beitragen. Letzteres erscheint wichtig, denn nach den Angaben von Klenk [12] über den Sättigungsgrad der Fettsäuren in Phosphatiden wäre bei ausschliesslicher Bindung des Osmiums in Form des Diesters nur ein Molekül Osmium pro Molekül Phosphatid unterzubringen.

Die 25–30 Å dicken, dunklen Streifen im elektronenoptischen Bild würden demnach durch die Ablagerung und Bindung von Osmium zwischen den Fettsäureresten der bimolekularen Lamellen in Höhe der Doppelbindungen hervorgerufen. Damit stimmt gut überein, dass synthetische Phosphatide mit gesättigten Fettsäureketten nicht mit OsO_4 reagieren [7].

Eine Aufklärung der Zusammenhänge zwischen der Struktur von bimolekularen Lipoidlamellen und elektronenoptischem Bild erscheint wichtig, da Lipoide ein weit verbreitetes Bauelement der Zelle darstellen. So zeigt die Markscheide des Nerven im Elektronenmikroskop ebenfalls die 25–30 Å breiten, dunklen Streifen [20], die wir nach dem Befund an Myelinfiguren wohl auf die Lipoidlamellen beziehen müssen. Da sich die Markscheidenstruktur und andere Membranen der Zelle in ganz ähnlicher Form auch nach Fixierung mit $KMnO_4$ darstellen lassen ([14], s.a. [19]), wäre es wichtig zu untersuchen, ob vom Mangan ähnliche Verbindungen bekannt sind, die zu einer Vernetzung der Lipoidmoleküle und damit Stabilisierung der Struktur von Lipoidlamellen führen könnten. In der Literatur haben wir nur die dem „Monoester" (I) der Osmiumsäure entsprechende Verbindung des Mangans gefunden [4].

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Abb. 1.—Myelinfigur im Cytoplasma eines Makrophagen der Kaninchenmilz. Bei den dichten Granula in der Umgebung der Myelinfigur dürfte es sich um die Eisenhydroxydmicelle des Ferritin handeln [22]. 150000×. Ausschnittvergrösserung 300000×.

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ACTION DE LA VITAMINE A SUR LA TRANSHYDROGÉNASE ET LA TPNH CYTOCROME *C* REDUCTASE

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Reçu le 31 juillet 1957

Il est vraisemblable que l'oxydation du triphosphopyridine nucléotide réduit (TPNH) dissipe plus de chaleur que celle du diphosphopyridinenucléotide réduit (DPNH); on sait, en effet, que le rendement de la phosphorylation oxydative est nettement plus faible pour l'oxydation du TPNH que pour celle du DPNH [9, 15, 16, 17]. L'accroissement du métabolisme basal mis en évidence au cours de la carence en vitamine A [4, 6, 2] peut s'expliquer par une modification d'activité des systèmes enzymatiques d'oxydation du DPNH et du TPNH. Afin de vérifier cette hypothèse les activités de la transhydrogénase, de la DPNH-cytochrome *c* réductase et de la TPNH-cytochrome *c* réductase ont été déterminées dans les mitochondries de foie de rats normaux ou carencés en vitamine A.

La carence en vitamine A a été pratiquée dès le sevrage chez des rats pesant entre 32 et 35 g. Un régime de base identique [3] est fourni à trois lots d'animaux. Les

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Tableau I

Activité spécifique de la transhydrogénase, de la TPNH-cytochrome c réductase et de la DPNH-cytochrome c réductase dans les mitochondries de foie de rats carencés ou non en vitamine A.

	Lot N° 1 carences (10 rats)	Lot N° 2 Vit. A 30 μg/jour (7 rats)	Lot N° 3 Vit. A 1000 μg/jour (7 rats)
Transhydrogénase			
(DPN réduit $\times 10^{-3}$ μM/min./mg azote)	29 ± 2[a]	44 ± 6	45 ± 6
TPNH-cyt. *c* réductase			
(cyt. *c* réduit $\times 10^{-3}$ μM/min./mg azote)	91 ± 10	41 ± 4	39 ± 5
DPNH-cyt. *c* réductase			
(cyt. *c* réduit $\times 10^{-3}$ μM/min./mg azote)	364 ± 36	400 ± 42	413 ± 38

[a] Déviation standard σ.

animaux du deuxième et troisième lot reçoivent *per os* chaque jour, respectivement, 30 et 1000 microgrammes de vitamine A; ils constituent les témoins de référence par rapport au premier lot. Les animaux sont sacrifiés après 5 semaines.

Les mitochondries de foie sont préparées selon Schneider et Hogeboom [14] et finalement mises en suspension dans de l'eau distillée. On laisse séjourner la suspension aqueuse de mitochondries pendant 30 minutes à 25°C. Ce traitement entraîne une perte importante de la réserve endogène en pyridine nucléotides sans altération appréciable de la transhydrogénase et des cytochrome *c* réductases; il permet donc de diminuer, dans l'évaluation de l'activité de la TPNH-cytochrome *c* réductase, l'erreur de surestimation due à la présence de la transhydrogénase et du DPN endogènes. A la suspension de mitochondries ainsi traitée on ajoute une solution de digitonine à raison de 3,3 mg de digitonine par mg d'azote mitochondrial. La transhydrogénase est évaluée par la méthode de Colowick et coll. [5] en présence de tampon trihydroxyméthyl aminométhane (Tris) 0,05 *M* pH 7,4, et de Cl_2Mn 5×10^{-4} *M*, la TPNH-cytochrome *c* réductase par la méthode de Haas et coll. [8] en présence de tampon Tris 0.05 *M* pH 7,4, la DPNH-cytochrome *c* réductase selon Mahler et coll. [11]. Les mesures sont effectuées au spectrophotomètre Beckman (modèle DU), température 23°C.

La vitamine A est dosée sur des prises aliquotes de tissu hépatique selon Meunier et Raoul [12]. La teneur en vitamine A par gramme de foie frais est la suivante : 1200 ± 300[1] microg. (lot N° 3), 30 ± 6 microg (lot N° 2), traces non dosables (lot N° 1).

Les activités spécifiques (micromoles $\times 10^{-3}$ substrat réduit $\times$ min.$^{-1}$ $\times$ mg^{-1} azote) de la transhydrogénase et des cytochrome *c* réductases sont rapportées dans le tableau I. Chez les rats carencés l'activité de la DPNH-cytochrome *c* réductase

[1] σ : déviation standard.

n'est pas sensiblement modifiée; par contre, l'activité de la transhydrogénase est diminuée de 33 %, celle de la TPNH-cytochrome *c* réductase est au contraire augmentée de plus de 100 %. L'activité de la DPNH-cytochrome *c* réductase étant nettement supérieure à celle de la transhydrogénase dans les mitochondries de foie on peut estimer que la proportion de TPNH oxydé *via* la TPNH-cytochrome *c* réductase est fonction du rapport :

$$R = \frac{\text{Activité de la TPNH-cytochrome } c \text{ réductase}}{\text{Activité TPNH-cyt. } c \text{ réductase} + \text{Activité Transhydrogénase}}.$$

Chez les animaux pourvus en vitamine A ce rapport est en moyenne 0,42, chez les carencés il est de 0,76.

Le quotient de phosphorylation pour l'oxydation du TPNH *via* la TPNH-cytochrome *c* réductase est au maximum 1 [16], il est de 3 pour l'oxydation du DPNH [10]. Le rendement de la phosphorylation oxydative pour l'oxydation du TPNH dans les mitochondries dépend du rapport R. Ce rendement reste faible chez les rats carencés puisque chez ces derniers une proportion importante de TPNH est oxydée par l'intermédiaire de la TPNH-cytochrome *c* réductase. La quantité d'énergie non récupérée par phosphorylation durant l'oxydation du TPNH et vraisemblablement dissipée sous forme de chaleur est donc plus grande chez les rats carencés que chez les rats normaux. Cette conclusion est en accord avec les observations concernant l'accroissement du métabolisme basal au cours de la carence en vitamine A.

Il est intéressant de noter que l'action de la thyroxine sur la transhydrogénase et la TPNH-cytochrome *c* réductase [13, 1] est opposée à celle de la vitamine A. Cette particularité entre dans le cadre de l'antagonisme entre vitamine A et thyroxine signalé par de nombreux auteurs [7].

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AN EFFICIENT TECHNIQUE FOR PREPARING THE RENAL GLOMERULUS FOR ELECTRON MICROSCOPY[1]

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Received August 1, 1957

One of the time-consuming problems in preparing specific cellular groups within a piece of tissue for electron microscopy is the actual location of that group of cells in the tissue block, then trimming it down to the tiny surface area required for obtaining good thin sections. This is the situation with the renal glomerulus. It is especially important when it is desired to evaluate the results of an experiment, and an adequate survey of many specimens is necessary to confirm the experimental result. The following is the technique devised in our laboratory for studying large numbers of renal glomeruli.

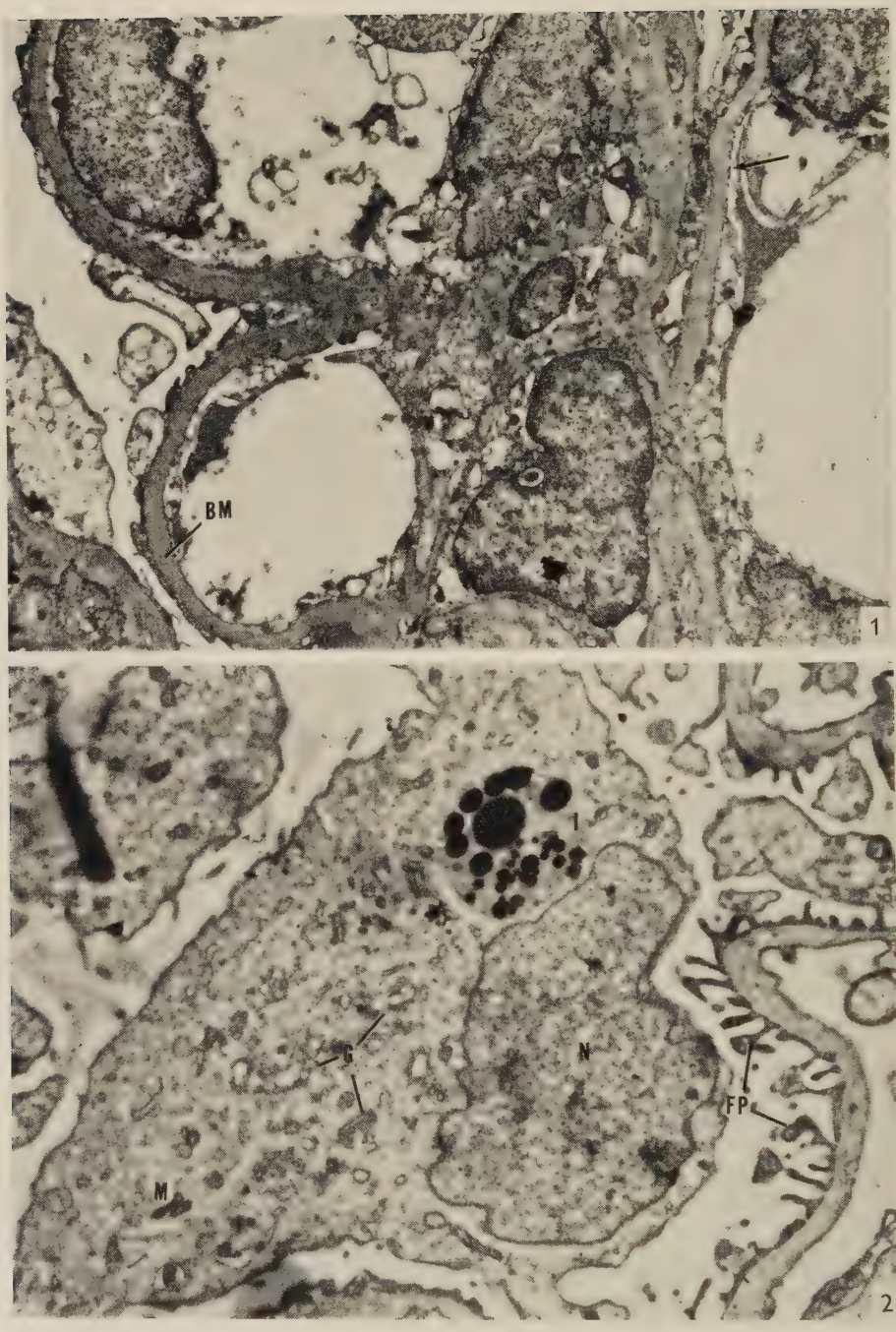
Method.—Fresh surgical specimens are removed from the kidney in small slices, approximately 1–2 mm thick. They are placed either in 1 per cent osmic acid, buffered to pH 7.4 with veronal acetate [4], or in 10 per cent formalin, buffered in the same way. Those placed in osmic acid are fixed for 1–2 hours, while the formalin fixed tissue is permitted to fix for several hours. The formalin fixed tissues are then rinsed in running water for one hour to remove the formaldehyde and are placed in a shallow dish of water under a dissecting microscope. With a fine dissecting needle, the blister-like Bowman's capsules on the surface of the slices are ruptured and the glomerular tuft fairly floats free with little manipulation. The glomeruli are collected with a pipette, and placed in buffered 1 per cent osmic acid for 1–2 hours, then rinsed with one or two changes of water.

The osmic fixed slices are handled in the same fashion, except that the dissected glomeruli are not again exposed to osmic acid. From this point on, the formalin fixed and osmic fixed glomeruli are handled in identical fashion.

A depression microscope slide is placed on a slide warming tray at 45°C, and melted 2 per cent agar is placed in each depression. The glomeruli are transferred to this melted agar with a fine pipette, taking care to carry over as little water as possible. The slides are removed from the tray, and the agar is sliced into tiny cubes after it solidifies, each cube containing many glomeruli. These cubes are then dehydrated and embedded as tissue blocks, following the description of Farquhar [3], and utilizing pre-polymerized methacrylate [5] and 70°C curing [1], the embedments are completed.

Discussion.—The actual dissecting process is far easier with formalin fixed tissue, since the glomeruli are more easily visualized, and are freed from the slice with less trauma. However, in some respects the fixation is slightly inferior to those fixed in osmic acid. The basement membrane proper, however, is always more sharply

[1] Supported by Grant #H-1725, National Heart Institute, National Institutes of Health.


BM
1
1
N
FP
M
2

defined in the formalin fixed post-osmicated specimens, and contrast is often greater, possibly due to leaching out of some protein.

Felix and Dalton [2] working with isolated cells recognized that this type of specimen is more susceptible to damage in the embedding than are tissue blocks and they devised the double embedding technique described above. In addition to reducing polymerization damage, the glomeruli are highly concentrated in a small block, facilitating sectioning of large numbers of specimens. The high temperature polymerizing technique [1], while used to reduce tissue damage, appeared to make only a small difference in the yield of undamaged glomeruli, compared to 45°C polymerization.

The technique described in this paper has been used primarily on human tissue, but it has proved successful on rabbits, cats, and dogs, even though the glomeruli are much smaller in these animals.

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SEX CHROMATIN IN EARLY CAT EMBRYOS

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Received August 2, 1957

The identification of genetic sex through the study of nuclear structure has been described by Barr and his associates [4, 5, 3, 7, 9] who have shown, in several different kinds of animals, that most of the somatic nuclei in female individuals possess a distinctive Feulgen-positive mass termed the "sex chromatin". This body was

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Fig. 1.—Renal glomerulus, human, 41-year-old female, fixed in buffered formalin and post-osmicated. Note high contrast, excellent preservation of basement membrane proper (*BM*), and well-defined discontinuity of endothelial lining (arrow). Philips Electron Microscope at 60 KV. Distance between bars, lower left, indicates one micron.

Fig. 2.—Epithelial cell from human renal glomerulus, 56-year-old male. Fixed in buffered osmic acid. Note good preservation of typical cell structures. Mitochondria (*M*) Golgi substance (*G*), and nucleus (*N*) are present. An inclusion body (*I*) is seen frequently in epithelial cells from human glomeruli. Note that although the foot processes (*FP*) of the epithelial cells are well-defined, the basement membrane proper is poorly preserved in this area. Philips Electron Microscope at 60 KV.

evidently lacking in most of the nuclei of males of the same species. In the neurons of adult females the sex-chromatin mass was characteristically disposed as a small sphere near or attached to the nucleolus, but in other cells it was plano-convex in shape and lay against the nuclear membrane. Graham [6] recorded sex chromatin in cat embryos, some of which were at stages of development before the visible differentiation of the gonads. The earliest embryos examined by Graham were recovered after 19 days' gestation. Recently, Park [10] has reported that he has investigated even earlier stages of development in human and monkey embryos; sex chromatin was detectable at 10–12 days of gestation and later, but not before this time.

Our investigations have yielded results similar to those of Park and lead to the conclusion that condensation of a sex-chromatin mass is unlikely in the large nuclei of early cleavage embryos.

Materials and Methods.—The material used was a series of cat embryos that had been collected and processed by one of us (E. C. A.) as part of a general investigation into embryonic development. The embryos were fixed with formalin or with Bouin's or Carnoy's solution, and stained with haematoxylin and eosin. The method used for the manipulation of the eggs and early embryos was similar to that described by Amoroso and Parkes [1].

Observations.—Altogether 311 embryos were available for examination: these ranged from 2-cell eggs to implanted embryos of about 20 days' gestation. In many embryos, however, fixation was unsuitable for the preservation of nuclear features, but there were 70 embryos in which the sex chromatin could be identified with reasonable certainty or its absence similarly established, and it is on these that this report is based. Where possible, 50–100 nuclei were examined in each embryo; in early cleavage stages all nuclei were scrutinised.

There were 29 implanted embryos (15–20 days' gestation) in which nuclear structure was well preserved, and in 15 of these the majority of nuclei contained the typical sex-chromatin mass. In the remaining 14 embryos few nuclei contained objects resembling the sex chromatin.

Of 12 blastocysts (6–14 days gestation), only one, a late blastocyst, exhibited sex-chromatin masses.

Twenty-six morulae presented clear nuclear pictures. The nuclei were characteristically round or oval in outline and contained one or sometimes two large nucleoli, the rest of the nucleus being occupied apparently by finely granular material. The nuclear membrane was well defined and there seemed little doubt that if the sex chromatin had been present in the typical form it would have been easily visible. Nevertheless, no such body was identified in any of the morulae.

Cleavage stages earlier than morulae were represented by only three embryos—one 2-cell, and two 4-cell eggs. In these, the nuclei were large and each contained several nucleoli. Some of the nucleoli lay freely within the nucleus but others were located on or near the nuclear membrane. Sex chromatin was not identified in any of the nuclei, but small nucleoli in contact with the nuclear membrane sometimes looked rather like sex-chromatin masses.

Discussion.—The chief finding in this investigation is that the "sex chromatin" of Barr *et al.* [4, 5, 3, 7, 9] could be identified in 15 out of 29 implanted embryos but not in any of the 26 morulae studied. This is in conformity with Park's [10]

observations, already cited, for it implies that the sex chromatin becomes recognisable during the second week of gestation. Sex chromatin appeared to be lacking also in the 2-cell and 4-cell eggs but its absence was not adequately demonstrated as only three such eggs were examined. In addition, the presence of small nucleoli, particularly in the nuclei of 2-cell and 4-cell eggs, would render recognition of sex chromatin extremely difficult. Such small nucleoli when lying on the nuclear membrane would, with their associated nucleic acids, tend to resemble the sex-chromatin mass both structurally and chemically. It appears, therefore, that even if the typical sex-chromatin mass were present in the very early embryo it would be unlikely to provide a reliable indication of sex.

A feature of the early cleavage stages is the progressive reduction in nuclear volume: Hertwig [8] showed that nuclear volumes are approximately halved at the second, third and fourth cleavage divisions. The nuclei of morulae are still perceptibly larger than those of well developed blastocysts and later stages. On the other hand, it is currently accepted that the nuclear content of deoxyribonucleic acid, the distinctive component of chromatin, remains constant in amount throughout development. Consequently, in the early phases of embryogenesis, the chromatin becomes constrained to progressively smaller nuclei. It is consistent that in rat eggs the nucleic acids associated with the nucleoli have been observed to form progressively larger masses, as nuclear size diminishes, in 2-, 4-, 8- and 16-cell stages [2]. Accordingly, we suggest that the sex chromatin fails to aggregate into its characteristic and recognisable form in morula nuclei because the larger surface areas available in these nuclei, as compared with the nuclei of late blastocysts and implanted embryos, permit the chromatin to become more sparsely distributed.

Summary.—The nuclei of cat embryos, from 2-cell eggs to implanted embryos of about 20 days' gestation, have been examined for the presence of sex chromatin. In all, 70 embryos have been studied in which nuclear structure was well preserved.

Sex chromatin was recognised in 15 out of 29 implanted embryos, in one out of 12 blastocysts, and in none out of 26 morulae. In one 2-cell and two 4-cell eggs several small nucleoli were seen, some of which lay on the nuclear membrane and presented an appearance similar to that of sex chromatin.

It is suggested that recognisable sex chromatin can only develop when the nuclei of the early developing embryo reach a sufficiently small size.

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NOTE ON NON-CHIASMA-TYPE ASSOCIATION BETWEEN THE X AND Y CHROMOSOMES OF *DROSOPHILA MELANOGASTER* AND *MUS MUSCULUS*

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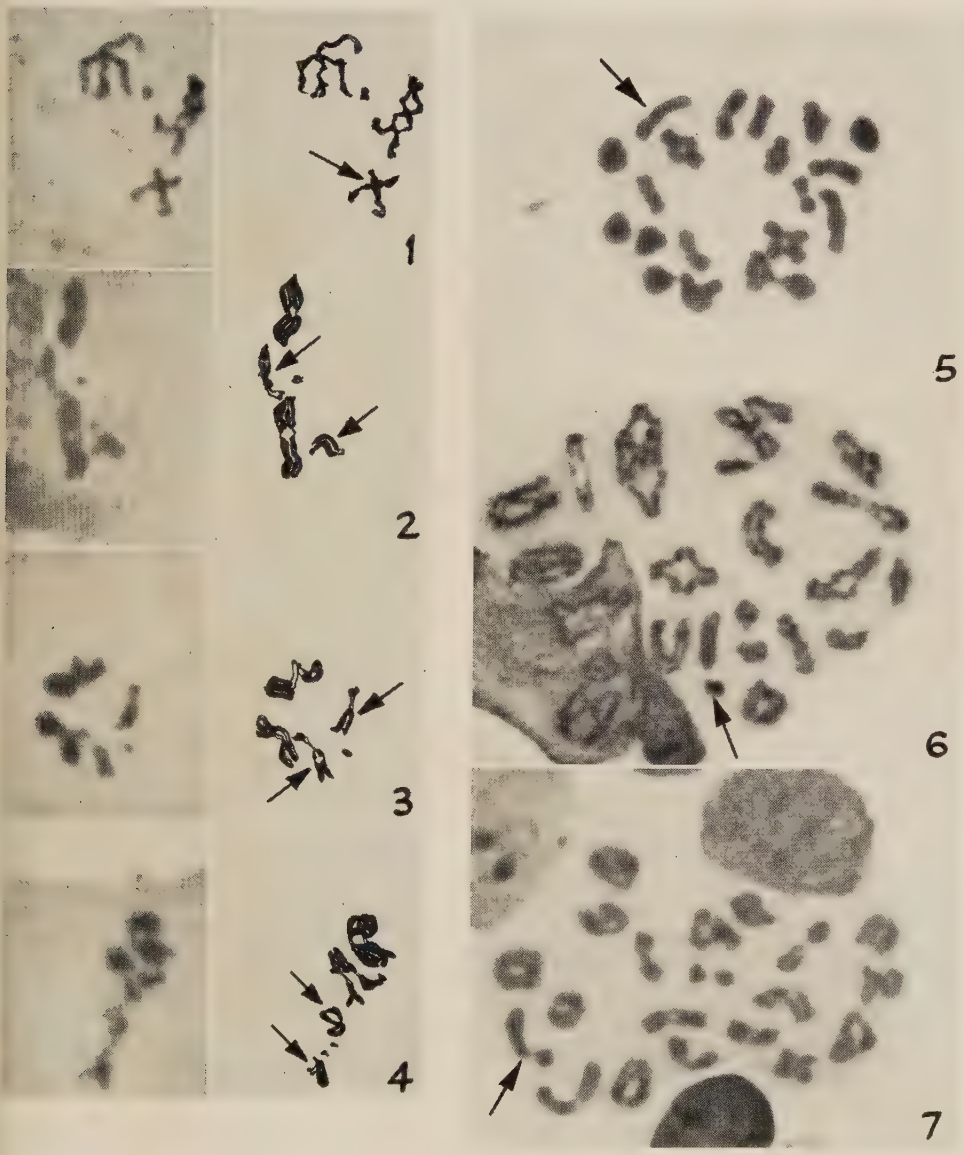
Received August 3, 1957

The X and Y chromosomes of the mouse, *Mus musculus*, each carrying a nucleolus-organizer, are not paired but held together by the nucleolus during meiotic prophase [5]. The end-to-end association of the X and Y observed at first metaphase appears to be due to means other than chiasmata [6]. The X and Y of *Drosophila melanogaster*, also carrying nucleolus-organizers [2, 3], are attached to the nucleolus during meiotic prophase [1]. They, at first metaphase, are believed to be held together by a nonchiasmate, yet paired, association of short homologous segments [1]. It appears that in the case of the mouse and the fruit fly there is a non-chiasma type of association between the X and Y, probably relating to the nucleolus. On the other hand, the observations to be reported herein suggest that there is a difference in the non-chiasmate type of association as observed in *Mus musculus* and *Drosophila melanogaster*.

Adult males of the Canton-S strain of *Drosophila melanogaster* obtained from the Department of Zoology, University of Indiana, and adult male mice of CBA, AKR and A Heston strains were used for this study. Paired testes taken from 4 6 Drosophila males were placed in a drop of 50 per cent solution of acetic acid placed on a glass slide. The resulting suspension was firmly pressed between the slide and a coverslip. The squash preparations thus obtained were stained by Feulgen's method. Similarly, squash preparations of mouse testes were made. Some of them were pretreated either with distilled water or with ribonuclease by the methods described previously [4].

The X and the Y of *Drosophila melanogaster* at diakinesis are always held together at points about a third of the full length of the X and about a fifth of the full length of the Y (Fig. 1). There may be short, mutually homologous segments at the point of association as described by other investigators [1]. The nucleolus-organizer of each chromosome is also located at about this point [2, 3]. As illustrated in Figs. 2, 3 and 4, the X and Y at the subsequent stages have not been observed as bivalents in the squash preparations; they always are seen as two separate univalents. The association of the X and Y appears to become very loose after diakinesis so that the chromosomes may be completely separated by simple squashing. On the other hand, the non-chiasmate, yet paired, association of homologous autosomes at first metaphase remains unbroken by squashing (Figs. 2, 3). It is unlikely, therefore, that the X and the Y at diakinesis and first metaphase are held together by the same forces that hold the homologous autosomes together. Considering the association of the X and Y to the nucleolus in meiotic prophase, the association of diakinesis is very likely due to the remnant of the nucleolar material. The further disintegration and loss of the nucleolar material may account for the weakening of the association at first metaphase.



Photomicrographs were taken by Leitz Panphoto 15 × 90. The XY bivalent and the separated X and Y are indicated by arrows.

Fig. 1–4.—From squash preparations of *Drosophila melanogaster* testes. *Fig. 1*, Diakinesis; *Fig. 2*, early metaphase; *Fig. 3*, polar view first metaphase; *Fig. 4*, side view first metaphase.
Figs. 5–7.—Late diakinesis or first metaphase figures of *Mus musculus* spermatocytes. *Fig. 5*, from a squash preparation; *Fig. 6*, from a squash preparation pretreated with distilled water. *Fig. 7*, from a squash preparation pretreated with ribonuclease.

The end-to-end association of the mouse X and Y at late diakinesis and first metaphase is not disrupted by simple squashing (Fig. 5). Moreover, it resists the pretreatment with distilled water (Fig. 6) and ribonuclease digestion (Fig. 7). The end-to-end association of the mouse X and Y at diakinesis and first metaphase appears to be as firm as the chiasma-type association of homologous autosomes.

Neither the autosomes nor sex chromosomes of the male of *Drosophila melanogaster* require chiasmata for metaphase association or for normal segregation, although bivalent formation appears to be necessary. Whatever the nature of the forces which hold the homologues together, the association is apparently more tenuous in the case of the X and Y than in the case of the autosomes. The X and Y at first meiosis may be held together by the nucleolus and its remnant. In the case of *Mus musculus*, however, some other means appear to be involved in the end-to-end association of the X and Y chromosomes.

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QUANTITATIVE HISTOCHEMISTRY BY MEANS OF RADIOACTIVE INDICATORS—ALKALINE PHOSPHATASE

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Received August 8, 1957

Numerous attempts have been made to develop quantitative procedures for the histochemical determination of enzymes in tissue sections [1]. From the point of view of accuracy, the most successful of these is probably that of Doyle [2] and Doyle *et al.* [3] for alkaline phosphatase [4, 8] in entire sections. But even this procedure does not appear to have been much used (although it should be applicable to a wide variety of enzymes), presumably because it is not convenient for routine studies.

In a previous publication [7] we demonstrated how quantitative Gram staining could be achieved by simple end-window counting of a smear on a slide, taking advantage of the fact that inorganic iodine is cooperatively bound in the Gram complex, and using ^{131}I-labelled Lugol solution. The general application of this principle to staining reactions was then pointed out and we have since been applying it on a wider scale. We shall here illustrate its application to the quantitative histochemical determination of alkaline phosphatase and its usefulness in studying the validity of some of the techniques in histochemistry.

In the Gomori Takamatsu procedure [4, 8] the enzymatically liberated phosphate ions are precipitated by calcium at the site of enzyme action. The precipitate is

converted, by exchange, to cobalt phosphate, following which the addition of ammonium sulfide results in the formation of a visible brown-black deposit of CoS. It is obvious that if labelled calcium, cobalt, sulphur or phosphate-labelled glycerophosphate were used in this reaction, the quantity of substrate hydrolyzed *in situ* on the slide could be measured by simple end-window counting.

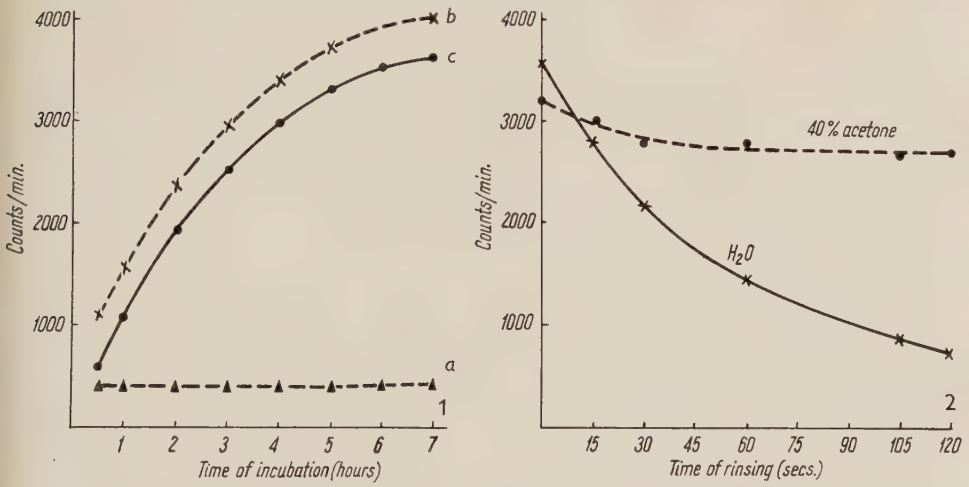

Fig. 1 *(left).*—Course of enzymatic hydrolysis of β-glycerophosphate by 6 μ section of kidney cortex, area 15 mm^2, incubated at 37°C in medium containing 2 μC ^{45}Ca/ml. (*a*) without substrate, (*b*) with substrate, (*c*) difference between curves *a* and *b*.
Fig. 2 *(right).*—Rate of removal of ^{45}Ca from an incubated section by rinsing with water or 40 per cent acetone. (Before the rinsing operation, sections incubated 2 hours in presence of 3 μC ^{45}Ca/ml.)

Initially we used $^{60}CoCl_2$, estimating the extent of the reaction following incubation. Subsequently we changed over to $^{45}CaCl_2$ in the incubation medium since the following steps in the Gomori–Takamatsu procedure are thus eliminated and one can also follow continuously the rate of hydrolysis of substrate on a single section merely by interrupting the reaction at suitable time intervals, counting under a window counter and then continuing the incubation.

Most of our initial work has been done with acetone-fixed, paraffin-embedded pork kidney cortex sections, the incubation medium being that normally used [6] and containing about 2 μC ^{45}Ca/ml. Curve *c* of Fig. 1 shows a typical curve of enzyme activity, corrected for labelled calcium accumulated by a control section incubated simultaneously without substrate. The "activity" of the control sections is due mainly to ^{45}Ca accumulated by the surface of the glass slide and can be kept low by careful attention to technique.

We have used a variety of other methods for following the course of the reaction, such as incubation with non-labelled reactants followed by exchange of the precipitated phosphate, calcium or cobalt with the corresponding labelled substance. Undoubtedly the simplest of all of these, and based on the technique used for Gram staining [7], is to deposit a drop of about 10–20 μl of ^{45}Ca-labelled substrate solution

on a section maintained in a covered Petri dish saturated with water vapour. At given time intervals the slide is removed, rinsed, counted and a fresh drop of substrate applied.

The value of labelled indicators is also clearly evident in an examination of the validity of the techniques used. For example, in the normal procedure "rinsing" in water is recommended following incubation [6]. Curve *a* of Fig. 2 shows what happens when this is done. It is clear that the rinsing procedure may be of crucial importance not only for estimation of the final CoS deposit in the normal Gomori–Takamatsu procedure, but especially for accurate localization of the sites of enzyme activity. If rinsing is done with 40 per cent acetone, a solvent used for incubation purposes by Fredericsson (quoted by Doyle [1]), the solubility of the calcium phosphate precipitate is considerably reduced and much less is washed out (curve *b*, Fig. 2). It is true that Fig. 2 represents an extreme case. For very high enzyme activities, with consequently larger deposits of calcium phosphate, the amount removed even during vigorous rinsing may be very much reduced. But for tissue sections with low activity, the rinsing step is of great importance.

By incubating sections with labelled calcium and then converting the resulting precipitate to cobalt phosphate with *unlabelled* cobalt, we have found that the conversion of calcium to cobalt phosphate (and consequently to CoS) is not quantitative, a fact suggested some time ago by Holter *et al.* [5] on the basis of model experiments. Exchange of calcium for cobalt is more than 90 per cent complete for low activities but less than 65 per cent for high enzyme activities.

By incubation of sections in a medium containing ^{45}Ca (or $^{32}PO_4$-labelled substrate) one can also readily study some of the kinetics of the reaction. We have been able in this way to examine the inhibitory effects of higher calcium concentrations quantitatively. By incubation of similar sections at different temperatures we have shown that the activation energy for hydrolysis of β-glycerophosphate is about 10 kcal/mole over the temperature range 20–40°C. It is quite probable that a study of the kinetics of hydrolysis of different substrates, together with autoradiography, may provide a practical procedure for classification of the various phosphatases *in situ*.

We are also studying the application of the above technique to acid phosphatases, using ThB as an indicator for lead; for the determination of phosphatases in microorganisms; for the localization of enzyme activity by incubation in a labelled medium, followed by autoradiography; for an examination of the influence of different fixation techniques, etc.

Finally it should be pointed out that the procedures here described should be of general applicability for the quantitative histochemical determination of other enzymes, as well as non-enzymatic substances such as nucleic acids, ascorbic acid, etc.

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THE β-GLUCURONIDASE CONTENT OF *DROSOPHILA* EMBRYOS

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Received August 10, 1957

Observations on regenerating mammalian tissues [4] and on neoplastic tissues [3] have suggested that increased β-glucuronidase activity is characteristic of normal and abnormal proliferations of cells. A study of the β-glucuronidase content of *Xenopus* embryos during their early development, however, failed to reveal any marked increase of this enzyme at periods when cell proliferations would be a predominant feature [2].

Compared with other sources the β-glucuronidase content of *Xenopus* embryos is relatively low, being about $\frac{1}{6}$ of that of mouse liver. *Drosophila* embryos, on the other hand, appear to possess a glucuronidase activity which is comparable to that of mouse liver. The higher β-glucuronidase activity of *Drosophila* embryos, compared with those of *Xenopus*, makes them a more favourable object in which to study the behaviour of this enzyme during development. In the case of *Drosophila* there is the added advantage of genetic control.

The dry weight of 1000 *Drosophila* embryos is between 2–3 mg. This means that most biochemical determinations will require fairly large numbers of embryos, unless special micro techniques are employed. In the present case about 1000 embryos were required for each determination. The procedure, described below, was designed to yield between 500–1000 eggs/hr. *Drosophila melanogaster* (an Oregon K stock) was used throughout the experiments. The flies used were between 8–14 days old and were stored in bottles containing food at 22°C. 15 bottles each containing about 150 pairs of flies were used. For egg collection the flies were transferred to empty bottles which were closed by watch glasses containing a layer of well moistened agar. At hourly intervals the watch glasses were removed and replaced by fresh ones. The eggs were removed from the lids, dechorionated with 1 per cent W/V sodium hypochlorite solution, washed with water, counted, placed on moist agar plates and allowed to develop at 22°C. Development was stopped after 1, 2 $\frac{1}{2}$, 4, 7, 10 and 18 hrs by freezing the embryos (− 20°C).

After a period in deep freeze (1 4 weeks) the embryos were homogenized in a small amount of distilled water (c. 1 ml) and the homogenate stood in a refrigerator overnight. The homogenate was centrifuged (5°C) at ca. 3000 × 9. The residue from the centrifuging was then resuspended in a small amount of distilled water and again centrifuged. The original supernatant and washing were combined and made up to a known volume, calculated so that each ml represented the extract from ca. 500 embryos. The residues were resuspended in the same volume of water.

The enzyme activity of these preparations, extract and residue, was measured by the colorimetric determination of 8-hydroxyquinoline formed by the enzymic hydrolysis of quinolyl 8-glucuronide. Details of this method are given elsewhere [1]. The activity of the preparations was expressed as the amount of 8-hydroxyquinoline

TABLE I

β-Glucuronidase activity of Drosophila *embryos.*
Activities expressed as μg 8.0 H quinoline liberated/hr/g dry wt. of original material.

Stage of development	Age (hr.)	Extracts			Residues			% Activity in extracts
		Mean activity	Range	*n*	Mean activity	Range	*n*	
Cleavage	1	1800	1045–2733	6	3590	2540–4654	3	50
Syncytial blastoderm	2½	2348	2154–2635	5	3415	3008–3962	3	68
Gastrulation and germ band extension	4	2030	1500–2870	7	3051	1654–4023	5	67
Primary histogenesis	7	2391	2070–2662	4	4100	3462–4723	3	60
Shortening of germ band; head involution	10	1913	1133–2385	5	3710	2510–4269	2	60
Hatching activity	18	2469	2283–2654	2	4085	—	1	61

liberated/hr/g dry wt. of the original material. The dry weight of the embryos was determined on separate samples.

The results are summarized in Table I. Clearly extracts of *Drosophila* embryos possess an appreciable glucuronidase activity. This activity remains practically constant throughout development. The results also show that a large amount (ca. 40 per cent) of the enzyme remains bound to the residues from which the water extracts were prepared.

Although cellular proliferation must be a marked feature of the initial stages of development of *Drosophila*, no change in the level of β-glucuronidase activity can be associated with this phase. The idea that raised β-glucuronidase activity is characteristic of cells undergoing division does not appear to apply to embryonic tissue. However, the presence of the enzyme in such tissues suggests that it is part of the normal enzymic equipment of relatively undifferentiated cytoplasm.

We are indebted to Professor C. H. Waddington for the interest he has shown in our work. The Advisory Committee for Medical Research for Scotland provided a grant. One of us (S. J. C.) was the recipient of a Macaulay Fellowship.

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A NEWLY OBSERVED CYTOPLASMIC COMPONENT, THE *PENTALAMINAR BODY*

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Received August 15, 1957

In the course of the investigation of various properties of several tissues, a previously unidentified cytoplasmic body has been observed. Because of its structure, which will be described in detail later, we have called it *pentalaminar body*. It is not a regular constituent of the cytoplasm but does occur with great enough frequency in certain tissues to indicate that it is a real structure. The studies reported here are based on material which was fixed in either Dalton's fixative [1] or Palade's fixative [3]. Embedding was in methacrylate. Thin sections were examined in the EMU 2C electron microscope.

The pentalaminar bodies have been observed in human epidermal cells grown in tissue culture (Fig. 3), macrophages in normal mouse lung (Fig. 2), and macrophages in mouse peritoneal cavity growing on an implanted filter (Figs. 1 and 5).

The structure of this body as visualized from sections cut in several different planes can be seen in the three dimensional drawing (Fig. 6). It is made up of a continuous dense membrane folded back upon itself to make either a regular or irregular cup shaped formation. A less dense material is contained between the outer and inner limits set by the continuous membrane. This less dense material is divided into two laminae by a central membrane of density and thickness identical with the outer limiting membrane. This central membrane can be seen to be continuous with the outer membrane in some places although it ends freely in others (Fig. 2). Five lamina can thus be counted in the body except in the region in which the outer membrane folds back upon itself. In some instances the spacing between the outer and central dense membranes is constant (Figs. 1, 3, 4, and 5); in others it is not (Fig. 2). The thickness of the outer and central dense membranes is about 80 Å, which places them within the size range of nuclear and ergastoplasmic membranes. The space separating the outer membranes has a fairly constant thickness of 300 Å. Because of the occasional serrated appearance presented by a cross section of the middle membrane, no constant dimension can be given for the distance separating it from the outer membranes. The shape of the bodies varies from that of a simple, cup shape to that of complex folds and invaginations. The overall dimensions have been observed to vary from about 0.2 μ to 8 or 10 μ, i.e., within the size range of mitochondria.

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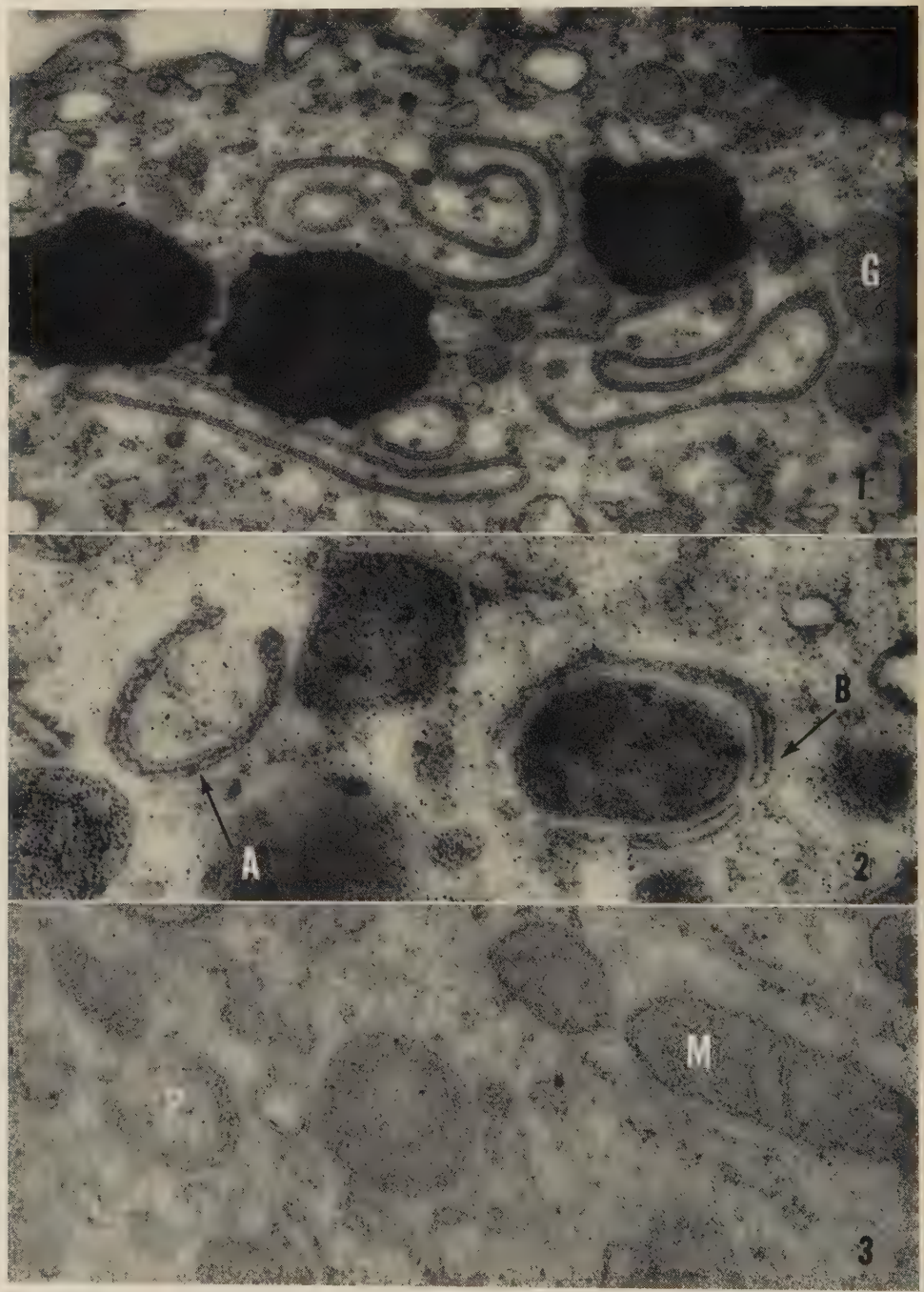
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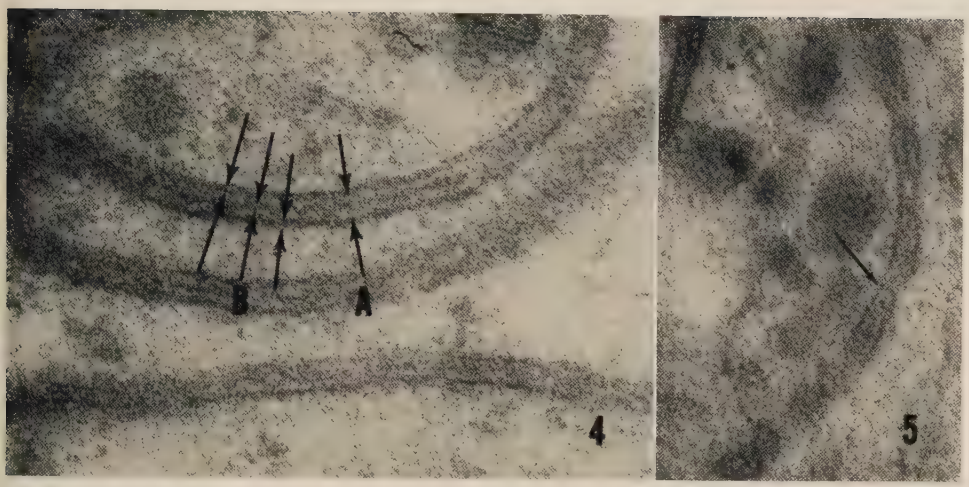

Fig. 1.—Macrophage growing on a filter implanted in mouse peritoneal cavity. Several pentalaminar bodies cut in cross section can be seen. It will be noted that the cytoplasm which appears to be surrounded by the pentalaminar bodies is the same as that elsewhere. Portions of four osmiophilic lipid droplets are seen, and also visible are several of the granular structures surrounded by a membrane which are characteristic of phagocytizing cells. *G* indicates one of them. × 30,400.

Fig. 2.—Macrophage from a normal mouse lung. The arrow at *A* indicates a place where one of the outer membranes becomes continuous with the central membrane of a pentalaminar body. The arrow at *B* points to the region where the central membrane terminates and where the outer membrane turns back upon itself. Just below and to the left of this point can be seen a section of a pentalaminar body which was cut between the termination of the central membrane and the point where the outer membrane turned back. The dense granules have tentatively been identified as ferritin [2]. × 61,800.

Fig. 3.—Human epidermal cell growing in tissue culture. A pentalaminar body at *P* cut so that the open part of the cup is visible. It may be compared in size to the mitochondrion *M*. × 61,800.

Fig. 4.—The arrows at *A* point out the space between the outer membranes. This space is about 300 Å thick. The arrows at *B* point out the thickness of the three dense membranes of the pentalaminar body. These membranes all measure about 80 Å in thickness. × 105,400.

Fig. 5.—The arrow points to the place where the outer membrane turns back upon itself in a pentalaminar body within the cytoplasm of a macrophage in the peritoneum of a mouse. × 77,900.

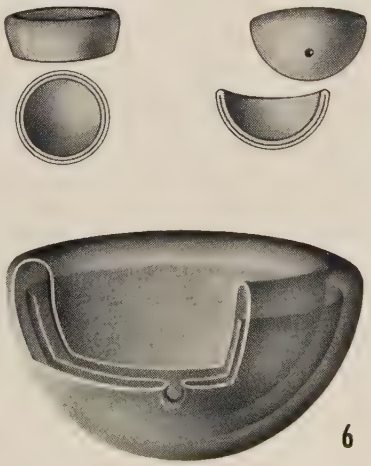

Fig. 6.—A three dimensional artist's drawing of the pentalaminar body as visualized from sections in different planes.

The total thickness of these structures is not great enough to resolve with the light microscope.

A functional significance cannot be assigned to the pentalaminar bodies with the knowledge available at present. However, a fact which may be of significance is that all of these bodies so far observed were in cells active in either phagocytosis or pinocytosis. Since our observations are necessarily limited it may be that cells not carrying out these functions also possess these structures, although we have not seen them in several other kinds of tissue cells which were carrying out different kinds of activities.

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UTILIZATION OF SERUM PROTEINS BY NORMAL AND POLIOVIRUS INFECTED MONKEY KIDNEY CELLS[1]

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Received August 19, 1957

The necessity for adding complex substances to tissue culture systems to promote fibroblastic outgrowth was first reported by Carrel [2]. He observed that the addition of embryonic tissue extracts accelerated the growth of chicken fibroblasts. Later Simms and Sanders [9] reported the successful use of serum ultrafiltrates as an additive to tissue culture fluid for certain systems. Sanford *et al.* [8] concluded that, although serum ultrafiltrate as well as serum residue supported cell proliferation, neither was as effective as whole serum. Allgoewer *et al.* [1] also reported that the addition of whole human serum to the medium was necessary to promote epidermal outgrowth of human skin *in vitro*, while gamma globulin as an additive showed no growth promoting activity. Kent and Gey [5] found that cultured cells of neoplastic origin utilized serum proteins.

It was the purpose of our investigation to determine quantitatively by means of electrophoretic analysis the patterns of serum utilization during the course of epithelial-like outgrowth of monkey kidney cells *in vitro* prior to and after infection with poliovirus.

[1] Aided by a grant from The National Foundation for Infantile Paralysis.

Methods and Materials.—Flat bottomed 8 ounce "prescription" bottles were seeded with 50 ml of a 1:200 suspension of trypsinized monkey kidney cells in a growth medium essentially the same as that described by Melnick [6]. Instead of the recommended 2 per cent calf serum, 10 per cent was used to increase the accuracy of the electrophoretic assay. The addition of this relatively large amount of serum did not interfere with epithelial-like outgrowth. The serum employed in all of these studies

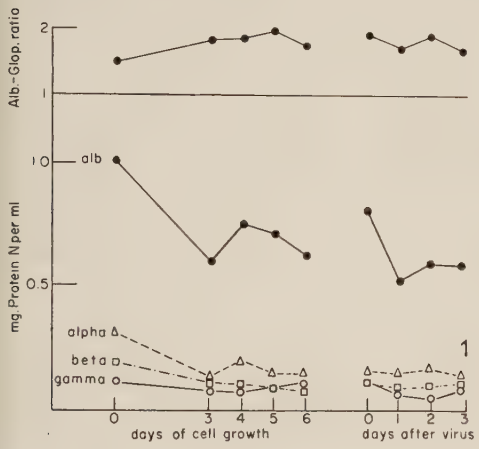

Fig. 1.—Fraction Protein N in mg/ml vs time before and after infection. Albumen–globulin ratios vs time before and after infection.

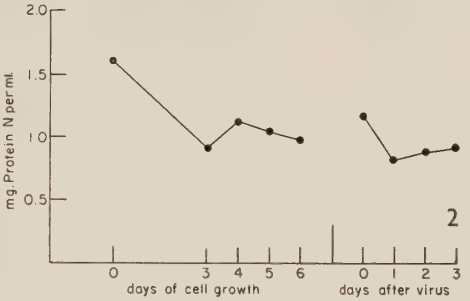

Fig. 2.—Total Protein N in mg/ml vs time before and after infection.

was obtained from a single calf and was inactivated for 30 min. at 56°C before use. The virus employed was the Mahoney strain of poliovirus containing 1×10^7 PFU (plaque-forming units) per ml, as determined by the plaque technique of Dulbecco [3]. Two milliliters of this virus fluid in 50 ml of the medium was used to infect the cells. Electrophoretic studies were performed at 1°C in a Perkin–Elmer apparatus equipped with the Philpot–Svensen optics. A 2 ml Tiselius cell was used in the standard open system. The solutions were equilibrated by dialysis for 14 hours under constant agitation at 4°C with diethyl barbiturate buffer at pH 8.5 to 8.6 and at an ionic strength of 0.1. All solutions were centrifuged at $105{,}000 \times g$ for one hour to remove particulate matter prior to dialysis and prior to Kjeldahl nitrogen determinations. The variations which took place in the serum protein as a result of cell growth were measured by comparison with the supernatant of a centrifuged sample from the cell suspension as used to seed the bottles. Changes which took place as a result of virus activity were measured by comparison with standard medium containing filtered virus fluid. For electrophoretic assays and for nitrogen determinations 8 ml samples were withdrawn from the respective bottle cultures without replacement of the fluid.

Results and Discussion.—The data in Fig. 1 which represent the average of 15 values show that during the first 3 days when epithelial like outgrowth was most

intense a decrease in albumin as well as in the globulins occurred. The increased albumin-globulin ratio indicates, however, a greater utilization of the globulins than of albumin. At this point the monolayers were well established but not confluent. During the fourth day an apparent increase from the 3 day level was observed for albumin as well as for alpha globulin. This increase was attributed to lysis of unattached cells and the subsequent liberation of soluble cellular proteins of high

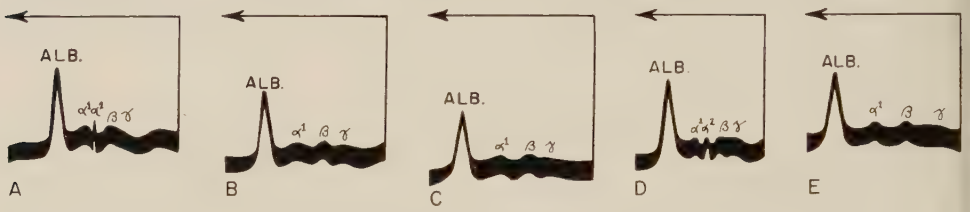

Fig. 3.—Electrophoretic patterns of different samples. *A*, Standard medium containing 10 per cent calf-serum; *B*, standard medium after exposure to cells for one hour; *C*, standard medium after 6 days of cell growth; *D*, standard medium containing filtered virus; *E*, standard medium 3 days after infection.

mobilities which migrated with albumin and alpha globulin. The presence of a significant number of non-viable cells in trypsinized monkey kidney cell preparations has recently been emphasized by Rappaport [7]. It was difficult to determine whether utilization of the serum proteins occurred from the fourth to the sixth day since significant amounts of proteins from lysed cells were present in the solution. After the sixth day the fluid was decanted, the cell layer washed twice with the standard medium, and then infected with poliovirus. During the first day after infection a sharp drop in albumin as well as the globulins was observed. This corresponds to the period when an accelerated metabolic activity is apparent in the infected cells as observed by Hydén [4]. There was a gradual increase in the serum proteins for the following 2 days. This increase was again attributed to the liberation of soluble cellular proteins from virus-induced cell destruction.

When total protein nitrogen values were plotted as a function of time a rapid decrease during the first 3 days of epithelial-like outgrowth became evident (Fig. 2). Calculations made from the electrophoretic data showed that of the total decrease in protein nitrogen, 49.7 per cent was due to a loss in the globulins and 44 per cent due to the loss in albumin. The possible utilization of the serum proteins during the second 3 days was offset by the release of cellular proteins.

Within the first day after infection a sharp drop in protein nitrogen was observed. Of the total decrease 33.5 per cent was due to the loss of albumin and 27 per cent due to the loss in globulins. This represents a much more rapid utilization of the serum proteins compared to that during normal cell growth. A subsequent gradual increase was again attributed to lysis of cells. As shown in Fig. 3 a difference was noted between the electrophoretic patterns of medium which had contained cells for approximately one hour prior to centrifugation and those of the medium only. In the medium without cells an alpha-2 globulin appeared consistently, whereas in the medium exposed to cells and then centrifuged no alpha-2 globulin separated. It

appears that the cells actively participate in the removal of the alpha-2 globulin. No boundary anomalies were observed in the electrophoretic patterns during the period of cell growth and after infection. Furthermore, the total amount of protein was higher in the sample exposed to cells for 1 hour than in the medium only. Although no fluid change was made during the course of cell growth, a continuous monolayer was formed. The cells retained their susceptibility to the virus since the supernatant fluid after infection and lysis of the cells contained 1×10^7 PFU per ml. The total protein nitrogen of the control medium without cells did not change during 6 days of incubation. Likewise no significant changes in the mobilities of the serum components either before or after infection were observed.

From the results obtained, several points emerge: all electrophoretically separable serum proteins were utilized by outgrowing epithelial like cells under the experimental conditions. It appeared that approximately 50 per cent of the globulin was utilized compared to 44 per cent of the albumin. Since a continuous monolayer of polygonal cells was obtained in six days, evidently a routine fluid change after 3 days of cell growth is not necessary to insure a confluent monolayer if a sufficient amount of serum is initially added to the medium. The yield of virus from cells grown under such conditions is identical to the one obtained in bottle cultures where the nutrient fluid was changed after 3 days of cell growth.

Summary.—The quantitative utilization of serum protein by epithelial-like cells in monkey kidney cell cultures *in vitro* has been studied. It was found that all electrophoretically separable serum components were utilized by the cells during the most active growth phase, namely the first 3 days. During the second three days of growth very little protein was utilized. After infection of the cells serum protein utilization was restricted to the first day, namely the period of virus multiplication. A continuous monolayer of epithelial like cells was obtained in 6 days without the 3 day routine fluid change. Since approximately half of the 10 per cent added calf serum was utilized it appears that the addition of 5 per cent calf serum would eliminate the necessity of a fluid change.

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A CORRELATION BETWEEN NUCLEAR ACTIVITY AND THE GROWTH PHASE IN CULTURES OF PROTOZOAN CELLS

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Received August 22, 1957

Few experimenters are unaware of the limiting conditions that arise when microorganisms are grown in culture flasks. Consequently, in order to obtain cells in the same physiological state, the logarithmic phase of growth is often used as the sampling period during which the experimental procedures are carried out. It is assumed that the constancy of the reproductive rate reflects the constancy of other cellular properties which are sustained throughout this period. All such work is, of course, a compromise, for the problem would hardly exist if chemostatic systems were routinely used [2]. It is difficult to understand how a closed system in which substrate exhaustion, growth-inhibiting by-products, and crowding effects are all increasing at a logarithmic rate could produce cells which are equifinal at all points of this phase of growth. Certainly at best the logarithmic growth phase must represent a period during which the cells are capable of compensating for these conditions within limits which are difficult to detect by cytological examinations.

Two independent observations which are pertinent to this problem are the subject of this brief note. One of us (L. S.) in the course of measuring the frequency distributions of cell volumes and the nuclear volumes in the protozoan ciliate *Tetrahymena pyriformis* found an interesting difference in the nuclear volume distribution between the logarithmic phase cells and stationary phase cells. The work includes distributions from three phases of growth; the lag phase, the logarithmic phase, and the stationary phase. The shapes of the distribution curves are the subject of a research program now in progress. The histograms, as would be expected, include a range of volumes which run roughly from daughter cell size to double this size for cells at prophase. The actual range rarely stays within these limits since the generation times, and consequently the cell volumes, show some variability. To determine the true range requires some knowledge of this variability. As a first approximation, however, one can readily find the most likely doubling range by accepting that range which includes the greatest percentage of the total sample. The curves can be analyzed further to give kinetic data on the growth of average single cells. The range and average values of all three distributions of nuclear and cell volumes are listed in Table I. A departure from this type of distribution is seen in the stationary phase of the nuclear volume which assumes a symmetrical bell shaped distribution with an average value of 3,200 μ^3. This is very near the values obtained for the daughter cell nuclear volume in both the lag and logarithmic phase cultures. It should be noted that the distribution of cell volumes at all three phases is comparable, as are the ranges of nuclear volumes in the lag and logarithmic cultures. The obvious interpretation of this finding is that the nuclear growth is more sensitive to inhibition

[1] Supported by California Cancer Grant No. 498.
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[3] Supported by National Science Foundation Grant No. 2490.

TABLE I

Cell volume and nuclear volume in Tetrahymena pyriformis *at different phases of growth.*

	Cell volume, μ^3		Nuclear volume, μ^3	
	Average	Range	Average	Range
Lag phase	50,800	33,000–66,000	4,500	3,050–6,100
Logarithmic phase	43,500	29,400–58,800	4,200	3,050–6,100
Stationary phase	48,300	33,000–66,000	3,200	—

than cell growth under these culturing conditions. The cell volumes in the stationary phase are distributed between 33,000 μ^3 and 66,000 μ^3 which is very nearly the same as found in the other two phases of growth. The lack of correlation between the change in nuclear and cellular volume could arise by either of two mechanisms. One mechanism would be through the inhibition of nuclear growth with the growth of cell volume continuing unimpaired. The other route might be normal nuclear and cell volume growth followed by a regression of nuclear volume to its daughter cell volume in the stationary period. A choice of one of these two alternatives will require more detailed work.

Samples of late logarithmic and stationary phase cells inoculated into fresh media will show the usual lag phase of growth. We propose that the length of lag phase of growth results in a large measure from the time required to bring nuclear growth from its daughter cell volume to its prophase volume, and consequently it is related to the generation time of the cell during its logarithmic growth. The observation which supports this contention is the synchronous bursts of divisions which are often seen at some period after the inoculation of a culture with stationary phase cells [1]. Further evidence comes from experiments conducted by one of us (E. B.) with the phytoflagellate *Chlamydomonas moewusii*. The lag phase in cultures of this organism comes to an abrupt end with an apparent burst of synchronous cell divisions. In many instances the duration of the lag phase approximates the generation time found in the logarithmic phase of growth. This will vary to some extent with the age of the culture used for inoculation. If the length of the lag phase and normal generation time are compared from cultures grown at different temperatures, a linear relationship is seen. Specifically, the plot of the length of the lag phase versus the generation time taken from cultures at a series of temperatures yields a straight line. This implies that the effect of temperature on these two variables of the growth curve is similar and could be interpreted to mean that they are, in fact, a measure of a single variable, the generation time. The low nuclear volume reflects a low nuclear activity during the stationary phase of growth and is the factor which tends to bring the cell nuclei to the same stage such that on reinoculation the period required for growth and synthesis to bring each cell to its prophase level of activity is the same and a synchronous burst of divisions results. We feel that these observations can have a definite meaning in evaluating various methods of synchronization of cell division.

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THE REFRACTION INCREMENT OF GLYCOGEN[1]

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Received August 22, 1957

A KNOWLEDGE of the specific refraction increment (α) of substances of biological importance is essential for the accurate determination of the solid concentration or dry mass of cells by refractometry or interference microscopy [2, 3, 4, 5, 7]. Tables of values for many substances have been given elsewhere [2, 3, 7] and on the basis of these we have suggested that an average value of 0.0018 be taken for protoplasmic solids [3]. This value appears to have been generally accepted. It is based on the assumption that the major intracellular constituent is protein. This is true in most cases, but in some cells large amounts of other substances, often in the form of discrete granules, may occur. Such a substance is glycogen and no detailed investigation of its refractile properties has been found in the literature. The refraction increment of many simple sugars such as glucose is about 0.00143, which is considerably lower than that of proteins. It was therefore decided to measure the refraction increment of glycogen over as wide a range of concentrations as possible.

Experimental.—The material selected was oyster glycogen (British Drug Houses Ltd.). Analysis showed 250 parts per million of nitrogen and 0.3 per cent sulphated ash. No reaction was given for reducing sugars. Preliminary experiments showed that this material formed concentrated opalescent solutions in water and a viscous gum having a refractive index of 1.382, corresponding to a concentration of over 30 per cent glucose, could be produced. It was thus felt that a maximum concentration of about 20 per cent of glycogen could be handled conveniently. (Excessive viscosity makes the accurate measurement of concentration very difficult.) 9·647 g of glycogen was weighed, placed in a 50 ml volumetric flask and distilled water added. The material was placed in a refrigerator for 24 hours and allowed to dissolve slowly without stirring. When solution appeared to be complete, the flask was made up to the mark with distilled water. A total of 44.12 ml of water was needed to produce 50 ml of solution. The partial specific volume of the material was thus calculated to be 0.61.

After careful mixing, samples were withdrawn for serial dilution and refractometry. Refractive indices were measured at 24°C in a Bellingham and Stanley Abbe type refractometer, using monochromatic mercury green light of wavelength 546 mμ. Indices were measured to four decimal places on several samples at each concentration. Sharp readings were obtained despite the opalescence of the solution.

Results.—The experimental results showing the relationship between the refractive index n and the concentration C (in g per 100 ml of solution) are plotted in Fig. 1. It will be seen that the relationship is linear within the limits of experimental error. The specific refraction increment can be calculated from the slope of the line. It was found to be 0.00134. However, this value requires correction for the water

[1] We are grateful to acknowledge the generous support of the Rockefeller Foundation and Smith Kline and French Foundation.

[2] Supported by a grant from the British Empire Cancer Campaign.

content of the material. This was kindly determined for us by Mr. M. P. Esnouf of the Department of Biochemistry, Oxford. The method used was vacuum drying to constant weight for 60 hours. A water content of 12.33 per cent was found. The corrected value of α for the dry material is thus 0.00150.

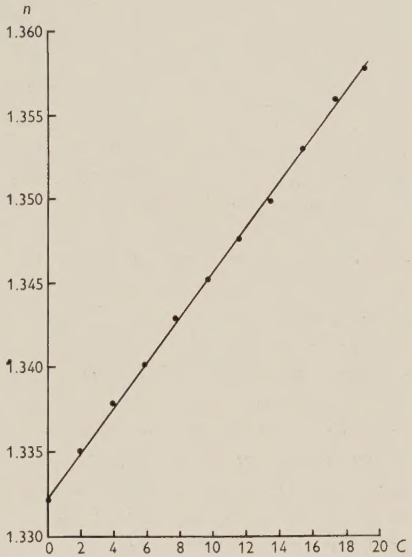

Fig. 1.—Variation of refractive index n with concentration C for glycogen solutions. The concentrations refer to the material as supplied and must be corrected for a water content of 12.33 per cent.

Discussion.—Refraction increments are usually measured for very dilute solutions. It is therefore of interest that the linear relationship between refractive index and concentration holds up to at least 20 per cent for glycogen and to about 50 per cent for bovine plasma albumin [3, 6]. The value 0.00150 for α is somewhat higher than that for glucose (0.00143), but it is comparable with values reported for dextran, namely 0.00151 [1] and 0.00152 [8]. It is well known that glycogens from different sources vary considerably in molecular size, but on general grounds it is unlikely that their refraction increments will differ greatly. Until more detailed figures are available, it is suggested that a value of 0.00150 should be taken for the refraction increment of intracellular polysaccharides.

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THE DETERMINATION OF THE WEIGHT OF BIOLOGICAL STRUCTURES BY ELECTRONS

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Received August 27, 1957

X-RAY micrography (microradiography) with polychromatic radiation permits the determination of the dry weight and water content of cytological structures [1]. In

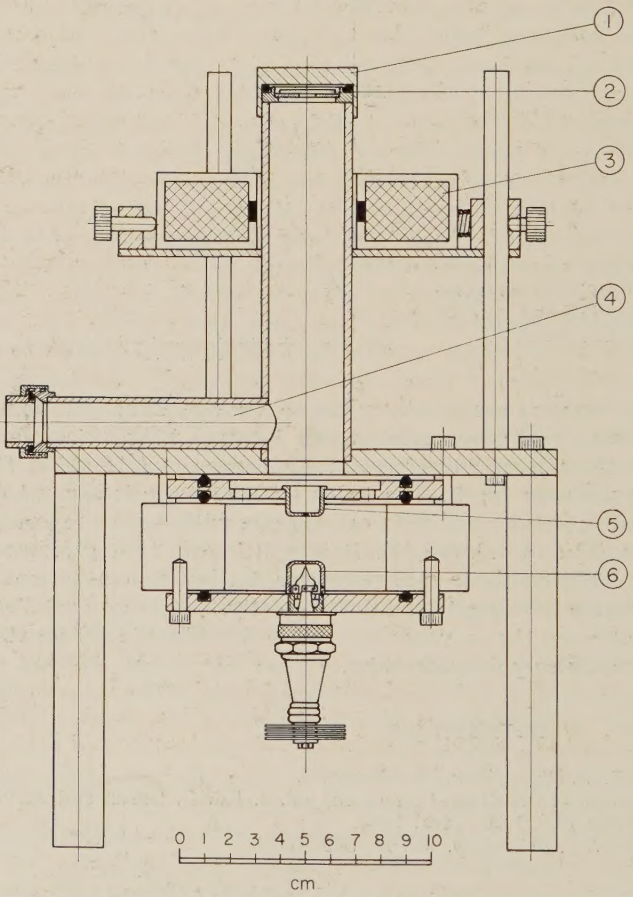

Fig. 1.—Cross section of electron source. *1*, Removable lid; *2*, sample-film; *3*, magnet; *4*, tube for evacuation; *5*, aperture-anode; *6*, cathode.

Fig. 2.—Optical enlargements of electron micrograms recorded at unit electron magnification. *A*, Section of a decalcified tooth, × 175. *B*, Section through an aortic wall, × 240. *C*, Section from a root tip (*Allium Cepa*), × 170. *D*, Smear of red blood cells (frog), × 350.

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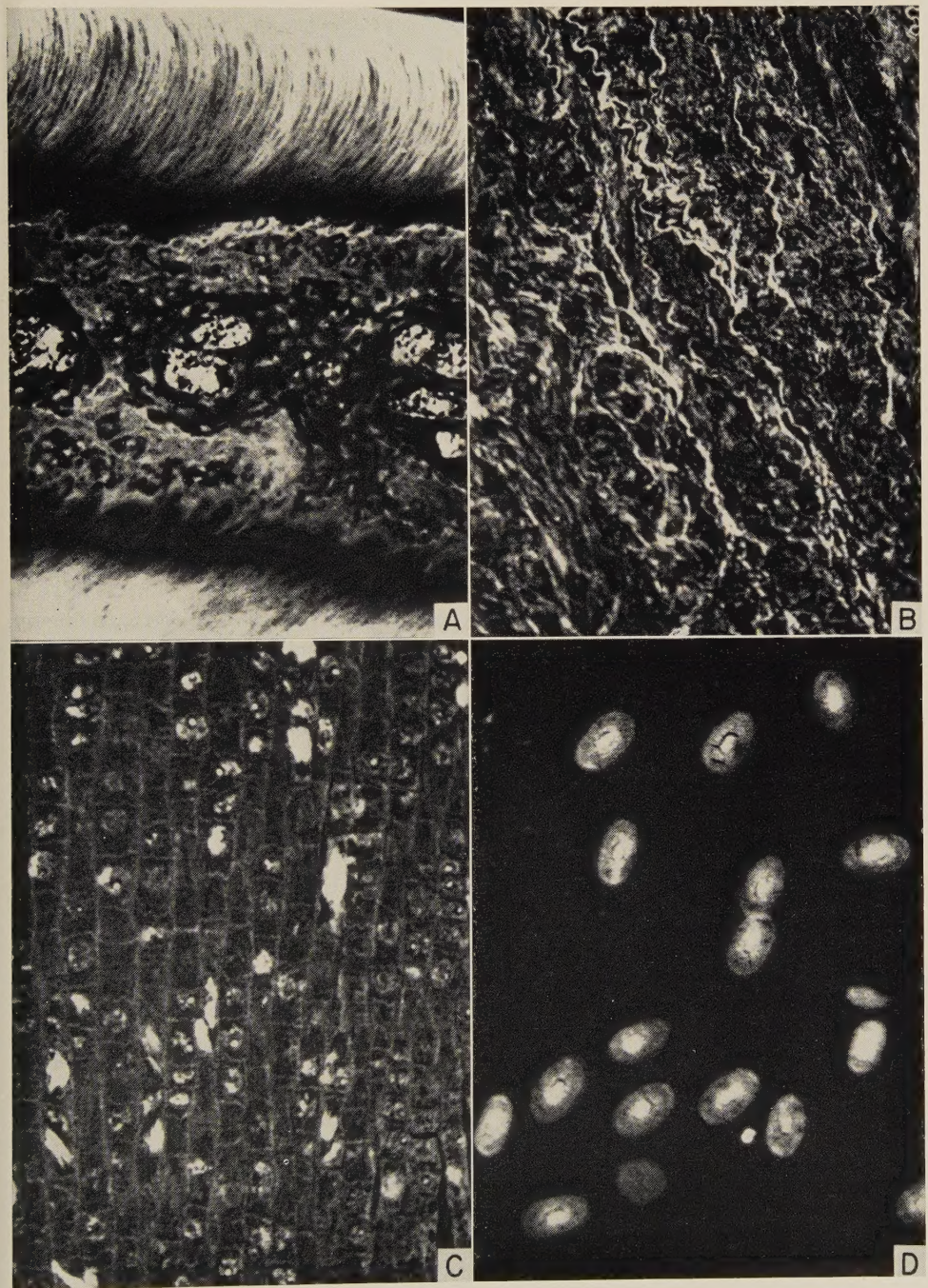
A
B
C
D

addition to this cytochemical X-ray weighing procedure the utilization of monochromatic X-rays permits elementary analyses of biological objects of microscopic size. The microradiographic weighing technique (X-rays) is based upon the fact that soft and ultrasoft X-rays are attenuated to about the same extent in the various components in the sample.

Fast electrons (X-rays) from radioactive sources are used to measure the amount of material (thickness) of various industrial products for example of paper during the production process. In the course of search for a technique for the measurement of the thickness and mass of biological samples a procedure based on the stopping of electrons in the biological microstructures has been developed.

The influence of atomic number on the stopping power of electrons varies only with a factor about 2 to 3 over the whole periodic system. Therefore, if electron micrograms are taken of a thin section of a biological tissue, it seems possible from the pattern of attenuation to evaluate the distribution of dry weight within the specimen. The geometry of image formation must be such that the intensity variations in the image are mainly caused by "absorption" in the specimen.

Some preliminary experiments have been performed in order to try to record microradiograms with electrons instead of X-rays. An electron source according to Fig. 1 generated a beam of electrons. The biological specimen was mounted directly on a fine grained photographic emulsion (Kodak Maximum Resolution Plate) in the same way as for X-ray microradiography and an electron transmission micrograph with unit magnification was recorded. The voltage by which the electrons have to be accelerated is relatively low in comparison with conventional electron microscopy. The suitable electron energy corresponds to $R = 3\ \varrho\ t$, where R is the range, and ϱ and t the density and thickness of specimen respectively. Approximately, $R = 1.46 \cdot E^{5/3}$, where E is the electron energy in Meo. A biological specimen a few micron thick has therefore to be imaged with 5 to 10 kV electrons in this modification of the weighing technique.

A resolution somewhat better than 1 μ has been obtained in our preliminary experiments with this simple technique, as is evident from Fig. 2. The exposure times are in general less than one second, with a low electron current.

A technique of weighing cellular structures by electron microabsorption measurements has certain advantages in comparison with the X-ray microradiographic technique, although the resolution of the former at present is not as good as of the latter. The absorption of the electrons is much less influenced by the atomic numbers of the elements of a composite absorber than what is the case for X-rays.

Further experiments are in progress in order to evaluate this simple form of "electron micrography" as a tool for cell chemical analysis.

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